The global dynamics of CDK substrate phosphorylation in a simplified CDK network

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Declaration

I Matthew Patrick Swaffer confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Cyclin-CDK complexes initiate S-phase and mitosis, but how these events are temporally separated is not fully understood. In *S. pombe* a single Cyclin-CDK is sufficient to order the cell cycle. Understanding how and when CDK substrates are phosphorylated, during the cell cycle, will inform how CDK orders cell cycle events. To this end, I have combined *in vivo* chemical inhibition of CDK and phosphoproteomics to study the behaviour of CDK-mediated phosphorylation.

Hundreds of CDK-dependent phosphorylation events have been identified and their cell cycle phosphorylation dynamics quantified. CDK substrates falls into three categories: i) the vast majority become phosphorylated during mitosis, ii) S-phase substrates, which are first phosphorylated at G1/S and are stable until mitosis, and iii) biphasic substrates that increases in phosphorylation at both transitions but peak at G2/M. All three classes are dephosphorylated simultaneously at mitotic exit.

S-phase substrates are more sensitive to CDK activity *in vivo* than mitotic substrates. The causal relationship between CDK activity, substrate phosphorylation, and cell cycle fate is corroborated by the fact that when the timing of substrate phosphorylation is reordered, the order of cell cycle events is correspondingly reorganised. These data argue that rising CDK activity orders the cell cycle via the attainment of substrate-specific thresholds. Low activity results in S-phase substrate phosphorylation, and the initiation of DNA synthesis, whilst higher CDK activity results in mitotic substrate phosphorylation and chromosome segregation. Furthermore, the timing of mitotic substrate phosphorylation during G2/M correlates with sensitivity to CDK activity, indicating that passage through sequential CDK activity thresholds orders substrate phosphorylation, and likely mitotic events, during the entry into mitosis.

Phosphoproteomic analysis also indicates that G1/S cyclins contribute mainly to the accumulation of CDK activity, and that phosphorylation by other mitotic kinases is organised into sequential waves, allowing the amplification and diversification of CDK signalling.

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Abbreviations

CDK	Cyclin-Dependent Kinase
CAK	CDK-Activating Kinase
DDK	Dbf4-Dependent Kinase
MPF	M-phase Promoting Factor
SAC	Spindle Assembly Checkpoint
APC/C	Anaphase Promoting Complex/Cyclosome (an E3 ubiquitin ligase)
SCF	Skp, Cullin, F-box containing complex (an E3 ubiquitin ligase)
MBF	MluI cell cycle box Binding Factor (a transcription factor complex)
SBF	Swi4/6 cell cycle box Binding Factor (a transcription factor complex)
CPC	Chromosome Passenger Complex
NEBD	Nuclear Envelope Break Down
PDB	Polo-Box Domain
SPB	Spindle Pole Body (yeast centrosome equivalent)
ORC	Origin Recognition Complex
MEF	Murine Embryonic Fibroblast
wt	wild type
AF	Cdc2 T14A, Y15F mutation
ts	temperature sensitive
HU	Hydroxyurea
Δ2 Δ13	c*dc2Δ, cdc13Δ*
ΔCCP	*cig1Δ, cig2Δ, puc1Δ*
SILAC	Stable Isotope Labelling with Amino acids in Cell culture
PCA	Principle Component Analysis
H:L	Heavy peak intensity to Light peak intensity ratio
min	minute(s)
h	hour(s)
Loc. Prob.	Localisation Probability
IC50	half maximal (50%) Inhibitory Concentration
AUC	Area Under Curve (i.e. integral of a graph)

Chapter 1. Introduction - Cyclin-Dependent Kinases and the control of eukaryotic cell division

"Omnis cellula e cellula"
Rudolf Virchow, 1855

"Cells. . . they make wise decisions and act upon them"
Barbara McClintock, 1983

Cell proliferation involves the coordination of cell growth and division, including the replication and segregation of a cell's DNA complement. The control over, and execution of, these processes underpins the proliferation of all living systems, but also ranks as some of the most dangerous and critical decisions a eukaryotic cell makes. The complexity of the eukaryotic cell and its genome necessitates a far greater level of control over the organisation of events during cellular division than that for prokaryotic life. More specifically, genomic fidelity during cell division requires the temporal separation of DNA replication, in S-phase, and chromosome segregation, at mitosis. The eukaryotic cell division cycle is driven by the protein kinase activity of multiple bipartite Cyclin-CDK complexes. Both the budding yeast, *Saccharomyces cerevisiae,* and the fission yeast, *Schizosaccharomyces pombe,* have a single CDK (Cdc28 and Cdc2 respectively) which were first identified in seminal screens in the 1970s for mutant alleles involved in cell cycle control, establishing these two organisms as model systems for cell cycle research (Hartwell et al., 1970, Hartwell et al., 1973, Nurse, 1975, Nurse et al., 1976). CDKs are Serine/Threonine kinases that are dependent on the association of the activating cyclin and are universal to eukaryotic cell division control, conserved from yeast to humans as was first demonstrated by the functional complementation of *S. pombe cdc2* with its human ortholog (Lee and Nurse, 1987, Nurse, 1990). Cyclin-CDK was subsequently demonstrated to be the enigmatic M-phase Promoting Factor (MPF) that peaks in mitosis and can initiate nuclear division when transplanted from an egg into an oocyte (Masui and Markert, 1971, Wasserman and Smith, 1978, Gerhart et al., 1984, Dunphy et al., 1988, Labbe et al., 1988a, Lohka and Maller, 1988, Labbe et al., 1989). The cell cycle control network of higher eukaryotic organisms is more complex than that of the yeasts and involves

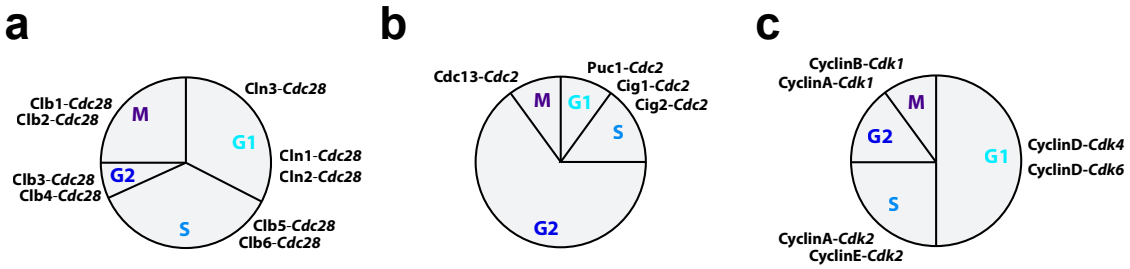

Figure 1.01 | Cyclin-CDK complexes drive different stages of the cell cycle
The Cyclin-CDK complexes and the respective cell cycle events they are associated with, in the model systems: **a** *S. cerevisiae* **b** *S. pombe* and **c** vertebrates.

multiple CDKs and cyclins (Santamaria et al., 2007, Malumbres et al., 2009, Satyanarayana and Kaldis, 2009). Different Cyclin-CDK complexes contribute to the orderly progression through different stages of the cell cycle, from yeast to higher eukaryotes (Figure 1.01).

Following the demonstration in the early 1980s that CDK is required for both mitosis and S-phase (Nurse and Bissett, 1981, Piggott et al., 1982) there have been a range of models proposed as to how CDK temporally organises the initiation of DNA replication and chromosome segregation (Hartwell and Weinert, 1989, Murray and Kirschner, 1989, Stern and Nurse, 1996). However, a coherent mechanistic picture of how the different biochemical properties and behaviours of Cyclin-CDKs order cell cycle events via the phosphorylation of their substrates is still largely lacking.

In the Introduction to this thesis, I will first outline the current understanding of how multiple regulatory parameters control the activity and expression of Cyclin-CDK complexes. I will then discuss the functional outputs of CDK activity in the control over DNA replication and mitosis and then how the dephosphorylation of CDK substrates, by counteracting protein phosphatases, is regulated. Finally, I will consider the conflicting bodies of genetic and biochemical data regarding how CDKs ensure the appropriate temporal organisation of the critical events of the eukaryotic cell division cycle.

1.1 The activation and regulation of CDK

CDK catalyses the phosphorylation of hundreds of target substrates, to bring about the events of S-phase and mitosis. The protein kinase activity of CDK is dynamically regulated over the cell cycle and requires the association between CDK and its binding partner cyclin. The first cyclin was identified in sea urchins as a protein whose levels oscillate, peaking during the mitosis of each cell cycle (Evans et al., 1983). Multiple cyclins have since been defined by their capacity to bind CDKs and the presence of the conserved cyclin box, which contributes to CDK association (Kobayashi et al., 1992, Noble et al., 1997). The mechanism of activation of CDKs has been informed by structural studies on the human S-phase Cyclin-CDK complex CyclinA2-Cdk2, during the 1990s. However iit is important to note that what is known for CyclinA2-Cdk2 might not always be reflective of other Cyclin-CDK complexes.

Monomeric and unmodified CDK proteins have no detectable kinase activity but can bind ATP (De Bondt et al., 1993, Solomon et al., 1990). Crystal structures of Cdk2 show the ATP binding site lies between the upper N and lower C-terminal domains, which are composed predominantly of beta-sheets and alpha-helices respectively (De Bondt et al., 1993) (Figure 1.02). The C-terminal domain contains a single highly conserved alpha helix (PSTAIRE helix) that binds directly with cyclin. Cyclin A2 binding to Cdk2 does not alter the cyclin tertiary structure but does drives major conformational rearrangements in the CDK molecule: the L12 helix is re-organised into a beta-sheet partially exposing the substrate-binding site, whilst the PSTAIRE helix also shifts toward the kinase upon cyclin binding (Jeffrey et al., 1995, Brown et al., 1995). In *S. cerevisiae* and *S. pombe* the chaperone Hsp90 (Cdc37) is important in Cyclin-CDK complex formation (Turnbull et al., 2006, Gerber et al., 1995, Farrell and Morgan, 2000, Mort-Bontemps-Soret et al., 2002).

The dynamics of CDK activity are highly non-linear as CDK activity rises during the cell cycle and is reported to peak abruptly upon mitotic entry (Draetta and Beach, 1988, Labbe et al., 1988b, Moreno et al., 1989). In early embryo extracts CDK

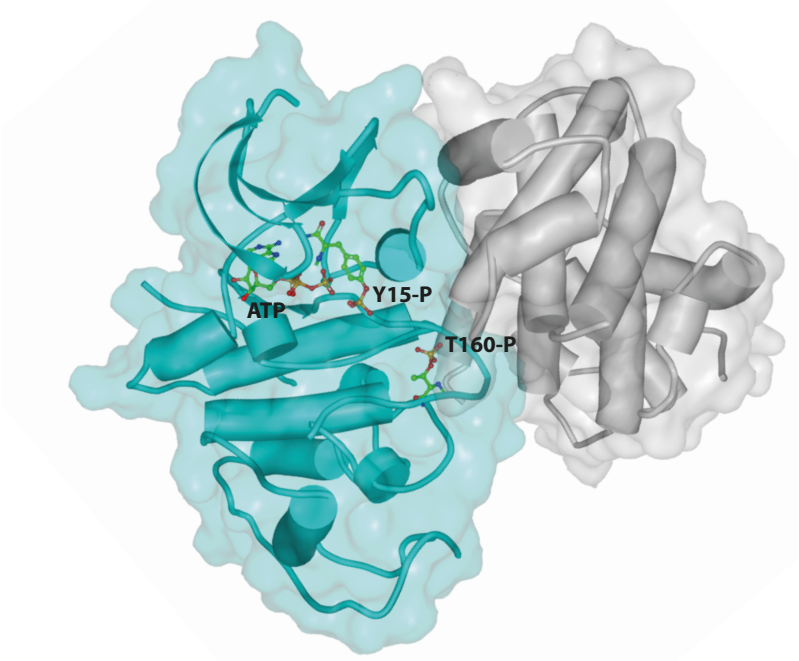

Figure 1.02 | Crystal structure of CyclinA-Cdk2-ATP (Y15-P, T160-P)
Cyclin A2 (grey) Cdk2 (blue) complex (PDB 2CJM).
ATP, phosphorylated Cdk2 Tyrosine 15 and phosphorylated Cdk2 Threonine 160 are shown and labelled.
Adapted from Welburn et al., 2007.

undergoes a switch-like jump in activity at a threshold concentration of cyclin, which occurs at mitotic entry. Once over this threshold, cyclin levels need to be reduced further than below the original threshold before CDK flips back into a low activity state, this property is called hysteresis and is reflective of the bistability in the system (Pomerening et al., 2003). Bistability likely ensures the system is robust to fluctuations and that once a cell commits to mitotic division the decision becomes irreversible. The activity of CDK is controlled at multiple levels; by the accumulation of the associating cyclins, regulatory phosphorylation, stoichiometric CDK inhibitors and subcellular localisation. The regulatory output of CDK is the net phosphorylation of its substrates and as such is a function of the activity of both CDK and CDK-counteracting phosphatases.

1.1.1 CDK activity is precisely regulated by phosphorylation

The association of cyclin with CDK alone is not sufficient to explain the dynamics of CDK activity (Pines and Hunter, 1989). Phosphorylation is another major regulatory input that controls CDK activity: CDK can be phosphorylated on three residues in a cyclin-association dependent manner: Threonine 14, Tyrosine 15 and at a conserved Threonine residue in the T-loop of the CDK (Cdc2-T167 in *S. pombe*).

1.1.1.1 T-loop phosphorylation activates and stabilises Cyclin-CDK

T-loop phosphorylation is essential for Cyclin-CDK activation, occurs after Cyclin-CDK association, at least for Cdk1, (Gould et al., 1991, Solomon et al., 1992) and enhances *in vivo* Cyclin-CDK complex stability *in vivo* (Larochelle et al., 2007) (Larochelle et al., 1998). Crystal structures of human CyclinA2-Cdk2 show that T160 phosphorylation re-positions the T loop from the catalytic cleft to fully expose the substrate binding site as well as re-orientating residues critical for ATP binding (Jeffrey et al., 1995, Russo et al., 1996b) (Figure 1.02). Phosphorylation of this conserved Threonine residue is brought about by the constitutively active Cdk-activating kinases (CAKs) (Kaldis, 1999). The major human and *S. pombe* CAK is Cdk7 (*S. pombe* Msc6), which itself is a cyclin dependent kinase that associates with Cyclin H (Msc2) and is also part of the TFIIH basal transcription factor (Fisher, 2005) (Larochelle et al., 2007) (Hermand et al., 2001). In *S. cerevisiae* Cdk7 is not involved in T-loop phosphorylation but instead a related but distinct monomeric kinase, Cak1, activates CDK through T-loop phosphorylation (Kaldis et al., 1996) (Cismowski et al., 1995). The *S. pombe* Cak1 ortholog can also phosphorylate Cdc2 *in vitro,* but its *in vivo* role is probably upstream of Msc6 (Hermand et al., 2001). CAK phosphorylation of CDK is dependent on cyclin association with CDK, except in *S. cerevisiae,* where the distinct CAK appears to have differing substrate requirements (Ross et al., 2000). The *Xenopus* Cdc2 T-loop is dephosphorylated during mitosis, and as such must be re-phosphorylated every cycle after novel cyclin synthesis and Cyclin-CDK complex formation (Lorca et al., 1992).

1.1.1.2 Tyrosine 15 phosphorylation negatively regulates CDK activity

The kinase Wee1 was the first described dose dependent inhibitor of mitotic entry (Nurse, 1975) (Russell and Nurse, 1987) and functions by catalysing inhibitory phosphorylation on CDK Tyrosine 15 (Y15) (and the adjacent Threonine 14 in some situations) (Featherstone and Russell, 1991, Parker et al., 1992, McGowan and Russell, 1993). Y15 is positioned on the roof of the ATP binding pocket and Y15 phosphorylation prevents the appropriate alignment of the bound ATP to transfer its gamma-phosphate to the substrate peptide (Welburn et al., 2007) (Figure 1.02). A second kinase also cooperates to phosphorylate Y15 with Wee1 in

fission yeast (Mik1) and higher eukaryotes (Myt1) (Lundgren et al., 1991, Mueller et al., 1995b). CDK Y15 phosphorylation occurs in interphase and is removed upon mitotic entry (Gould and Nurse, 1989). Cdc25 antagonises Wee1 to promote mitotic entry (Russell and Nurse, 1986) by dephosphorylating CDK Y15 (Dunphy and Kumagai, 1991, Gautier et al., 1991, Kumagai and Dunphy, 1991, Strausfeld et al., 1991). Humans have three Cdc25 isomers (Cdc25A-C) that can function to activate Cyclin-CDK during the cell cycle (Boutros et al., 2006).

1.1.1.3 Multiple signalling pathways regulate CDK Tyrosine 15 phosphorylation

Wee1 and Cdc25 act as signalling hubs to process multiple upstream inputs, allowing them to converge upon CDK activity and integrate cell intrinsic and extrinsic information into the decision to undergo mitosis. For example in *S. pombe* the, so-called, cell geometry sensing pathways includes the Cdr1 and Cdr2 kinases, which phosphorylate and inhibit Wee1, and are in turn phosphorylated and inhibited by the tip-localised kinase Pom1. This led to the proposal of a cell-sizing model where a gradient of Pom1 inhibits medial Cdr1/2, allowing Wee1 activity only in small cells when polar Pom1 and Cdr1/2 overlap (Martin and Berthelot-Grosjean, 2009, Moseley et al., 2009, Hachet et al., 2011). This pathway clearly modulates the timing of nuclear division via Wee1 but what information it actually conveys to CDK is not clear given that *pom1*Δ cells are still able to appropriately coordinate growth and division, albeit with a reduced size at mitosis (Wood and Nurse, 2013). Cdc25 activation is promoted by the spindle pole body (SPB) localisation and activation of the Polo-like kinase (Plo1) in *S. pombe* (Mulvihill et al., 1999, MacIver et al., 2003). Changes in nitrogen quality are monitored via Tor2 (TORC1) that in turn regulates the activity of the MAP kinase Sty1 (Petersen and Nurse, 2007). Increased Sty1 activity results in Plo1-S402 phosphorylation, promoting Plo1 recruitment to the SPB and in turn activating Cdc25 to bring about earlier mitotic onset when nitrogen is limiting (Petersen and Hagan, 2005). Sty1 also works downstream of a number of glucose sensing/cAMP signaling proteins that influence the timing of mitotic commitment (Navarro and Nurse, 2012).

One of the most important and ubiquitous cell-intrinsic signals that impinges upon

CDK Y15 phosphorylation is the surveillance of genomic integrity. In *S. pombe* the PIKK checkpoint kinase Rad3 (ATR) activates effector kinases Chk1 and Cds1 in response to DNA damage and stalled replication forks respectively (Walworth et al., 1993) (Rhind and Russell, 2000). In higher eukaryotes this role of Rad3 is split between ATM and ATR but still signals via the Chk1 and Chk2 (Cds1 ortholog) (Rhind and Russell, 2012). In fission yeast Chk1 and Cds1 phosphorylate Cdc25 at multiple sites to inactivate Cdc25, for instance by generating a docking site for 14-3-3 proteins (Rad24 and Rad25) that drives the nuclear exclusion of Cdc25 (Walworth et al., 1993, Lopez-Girona et al., 1999, Karlsson-Rosenthal and Millar, 2006). Across eukaryotes there are comparable mechanism of Chk1/Chk2-dependent control of Wee1 and Cdc25 localisation/proteolysis/activity that function to impose a cell cycle arrest downstream of DNA damage signalling (Karlsson-Rosenthal and Millar, 2006, Reinhardt and Yaffe, 2009). Monitoring of DNA damage and on-going DNA replication is also signalled through the Mik1 kinase in *S. pombe* but Mik1 is insufficient to produce a robust checkpoint arrest by itself (Rhind and Russell, 2000). In *S. cerevisiae* Cdc28-Y19 aligns with Y15 of other CDKs but is both non-essential and dispensable for checkpoint arrests in response to DNA-damage or on-going DNA replication (Sorger and Murray, 1992, Amon et al., 1992). However, Cdc28-Y19 phosphorylation by Swe1, the Wee1 ortholog, is responsible for the morphogenesis checkpoint, which delays mitosis when polarised bud growth is disrupted (Lew and Reed, 1995, Lew, 2003).

1.1.1.4 CDK activity promotes Tyrosine 15 dephosphorylation

As well as regulating CDK activity, Wee1 and Cdc25 are in turn regulated by CDK through a double negative feedback loop, in which CDK phosphorylates and inactivates Wee1, and a positive feedback loop, in which CDK phosphorylation activates Cdc25. (McGowan and Russell, 1995, Mueller et al., 1995a, Kumagai and Dunphy, 1992, Hoffmann et al., 1993, Okamoto and Sagata, 2007, Lu et al., 2012). In Xenopus, Wee1 and Cdc25 phosphorylation is highly ultrasensitive as a function of CDK activity, meaning that their phosphorylation responds to CDK activity over a narrow range of activities in an abrupt, step-wise manner (Kim and Ferrell, 2007, Trunnell et al., 2011). The combination of feedback and ultrasensitivity allows CDK

activity to switch between two steady states, explaining the highly non-linear and bistable dynamics of CDK activation at mitotic entry (Novak and Tyson, 1993).

The CDK-Cdc25-Wee1 feedback system is important for sustained and robust oscillations in CDK activity. T14AY15F mutations in CDK, which bypass this auto-regulatory network, result in attenuated oscillations in CDK activity in *Xenopus* extracts and the precocious termination of G1 as well as failure to appropriately complete mitotic events in HeLa cells (Pomerening et al., 2005, Pomerening et al., 2008). CDK activity, as measured by a phosphorylation FRET reporter, shows that T14A/Y15F CDK activity can undergo multiple attenuated oscillations that fail to properly initiate nuclear envelope breakdown (NEB) before reaching a CDK activity that is sufficiently high to initiate nuclear division. The rise in CDK(T14A/Y15F) activity, as it approaches the mitotic threshold, is slower and more linear than for a wild-type CDK (Gavet and Pines, 2010a). A Y15F substitution in *S. pombe* Cdc2 results in premature and pathological entry to mitosis (Gould and Nurse, 1989). Surprisingly viable fission yeast strains harbouring this mutation can be engineered when the CDK network is simplified (Coudreuse and Nurse, 2010). This network of feedback loops also allows CDK activation to behave as a trigger wave, facilitating the rapid spatial propagation of the mitotic state (Chang and Ferrell, 2013). This is of particular physiological relevance in cells in which events need to be coordinated over sizable distances, such as the *Xenopus* egg that is greater than a millimeter in diameter. In *S. pombe* the polo like kinase (Plo1) has also been implicated in the CDK activating feedback loop and is in turn positively regulated by CDK activity (Tanaka et al., 2001, Mulvihill et al., 1999, MacIver et al., 2003).

1.1.2 Proteolysis and transcriptional feedback loops generate oscillations in cyclin levels across the cell cycle

In addition to the phospho-regulation of CDK, sequential waves of cyclin expression coordinate the rise in CDK activity during the cell cycle. Robust oscillations in cyclin expression are generated via a series of nested positive and negative feedback loops.

1.1.2.1 The regulation of cyclin expression in budding yeast

The expression of the nine different *S. cerevisiae* cell cycle cyclins has been extensively studied over the last three decades and serves as a model example of how transcription factors (such as SBF and MBF) and ubiquitin targeted proteolysis (e.g. downstream of the E3 ubiquitin ligases APC/C and SCF) contribute to such waves of cyclin expression.

The G1-phase cyclins (Cln1-3) are involved in the G1 events of bud emergence and SPB duplication, as well as initiating a transcriptional program to drive the subsequent expression of Clbs (B-type cyclins). Of the six Clbs in *S. cerevisiae* Clb5&6 protein levels rise first and initiate DNA replication, followed by Clb3&4 then Clb1&2, which associate with Cdc28 to initiate mitosis. Destruction of Clb-Cdc28 then drives cells through mitotic exit. Both transcription and ubiquitin mediated degradation coordinate these sequential waves of cyclin expression (Morgan, 1997, Mendenhall and Hodge, 1998, Bloom and Cross, 2007). G1 cyclins, in the first instance only Cln3, inhibit the transcriptional repressor Whi5 allowing SBF and MBF to transcribe ~200 genes including the G1 cyclins Cln1&2, Cln1&2 then further activate their own expression as part of a positive feedback loop (Johnson and Skotheim, 2013, Bertoli et al., 2013). The dilution of a fixed amount of Whi5, against a constant concentration of Cln3, has been proposed to form the basis of a G1 size homeostasis mechanism in budding yeast (Jan Skotheim, personal communication). Cdc28-mediated phosphorylation of Cln-Cdc28 complexes then provides a binding site for F-box phospho-degron recognition proteins (Grr1 and/or Cdc4) to target Cln1-3 for SCF-mediated ubiquitination and proteasomal degradation (Lanker et al., 1996, Skowyra et al., 1999, Landry et al., 2012).

The G1/S peak in Cln1/2 SBF-dependent transcription is followed by sequential waves of Clb transcription, dependent at first on MBF (Clb5&6), which is subsequently repressed by Nrm1, and later on Fkh2-Mcm1 and Ndd1 transcription factors (Clb1-4). Rising Clb levels later in the cycle repress SBF to inhibit G1 cyclin expression and phosphorylate Fkh2 and Ndd1 to promote mitotic Clb expression (Bloom and Cross, 2007) (Bertoli et al., 2013). Clb6 is degraded by the SCF in a manner analogous to the Clns (Jackson et al., 2006) whilst the APC/C targets all

other B-type cyclins for ubiquitin-mediated proteolysis (Shirayama et al., 1999, Wasch and Cross, 2002). The APC/C, complexed to its co-activator Cdc20, is activated during early mitosis by Clb2-Cdc28 phosphorylation (Rudner and Murray, 2000) but later in M/G1 Cdc20 exchanges for a second co-activator, Cdh1, which alters the substrate specificity of the APC/C and completes the degradation of mitotic cyclins (Visintin et al., 1997). Cdh1 is phosphorylated and repressed by Clns and Clbs so APC/C^{Cdh1} only becomes active during mitotic exit and in early G1 (Zachariae et al., 1998, Jaspersen et al., 1999). S-phase Clbs are less sensitive to APC/C^{Cdh1} so the exchange of Cdc20 for Cdh1 also allows the re-accumulation of Clb5 during the subsequent G1/S, which in turn can phosphorylate and repress Cdh1 to allow the re-expression of mitotic B-type cyclins during the subsequent cycle (Yeong et al., 2001). Recently, small temporal differences in the timing of APC/C substrate proteolysis have been reported in budding yeast, for example Clb5 is degraded before Clb2 (Lu et al., 2014). APC/C co-activators recognise APC/C substrates via D-box or KEN box motifs (Glotzer et al., 1991, Pfleger and Kirschner, 2000) but the early degradation of Clb5 has been attributed to the newly identified ABBA motif as well as Cks1-mediated recruitment of Clb5-Cdc28-Cks1 to phospho-docking sites on APC/C (Lu et al., 2014). The precise significance of these timing differences is not yet clear.

1.1.2.2 Fission yeast cyclin expression is less complex

A more limited body of work in *S. pombe* suggests fission yeast cyclin expression is regulated in a related but perhaps simpler manner to budding yeast. *S. pombe* has four cyclins that differentially associate with CDK during the cell cycle: Puc1, Cig1, Cig2 and Cdc13 (Booher and Beach, 1988, Hagan et al., 1988, Bueno et al., 1991, Bueno and Russell, 1993, Connolly and Beach, 1994, Martin-Castellanos et al., 2000). The G1/S cyclin Cig1 is degraded at anaphase by the APC/C, increases again at the subsequent G1/S and is maintained at relatively unchanged levels until the next mitosis (Blanco et al., 2000). Cig2 is the major G1/S-phase cyclin in fission yeast (Mondesert et al., 1996) and is the only *S. pombe* cyclin that is strongly transcriptionally regulated during the cell cycle (Rustici et al., 2004). Cig2 transcript and protein levels peak at G1/S and then decline during G2 (Connolly and Beach,

1994) (Yamano et al., 2000). Cig2 is transcribed downstream of the *S. pombe* MBF transcription factor but also inhibits MBF as part of an auto-regulatory feedback-loop by binding one MBF subunit (Res1) and phosphorylating another (Res2) (Ayte et al., 2001). This negative feedback loop works either via or alongside the E3 ubiquitin ligase $SCF^{Pop1/Pop2}$, which also regulates Cig2 levels by reducing transcript levels (Yamano et al., 2000). Cig2 protein stability is decreased by the SCF during G2, in part dependent on the cullin Pop1 or F-box protein Pop2 (Yamano et al., 2000, Yamano et al., 2004). If SCF mediated proteolysis of Cig2 in G2 is bypassed, the degradation of Cig2 in mitosis by APC/C becomes essential for cell viability (Ayte et al., 2001). The major mitotic B-type cyclin Cdc13 starts to rise at G1 and peaks at G2/M before being degraded by the APC/C at anaphase, an event essential for mitotic exit (Moreno et al., 1989, Yamano et al., 1996). Presumably the *S. pombe* APC/C in mitosis is bound to the Cdc20 co-activator ortholog; Slp1, as in other organisms. The non-essential Cdh1 ortholog in *S. pombe*, Ste9/Swr1, promotes G1 specific degradation of Cig1 and Cdc13 but is also inactivated by CDK phosphorylation during the rest of the cycle because phosphorylation triggers its proteolysis and dissociation from APC/C (Blanco et al., 2000, Yamaguchi et al., 2000).

1.1.2.3 The regulation of cyclin expression, from yeast to humans

Within the networks that controls cyclin expression and degradation in budding and fission yeast there are numerous examples of a given cyclin regulating the transcription or proteolysis of its own or other cyclin's mRNA or protein. This underscores the prevalence of crosstalk, positive feedback and negative feedback in the control of expression of cell cycle machinery. Much of the network architecture and the principles of proteolysis and transcriptional regulation are present in the current understanding of the control over cyclin expression in higher eukaryotic cell division (Cross et al., 2011) (Pines, 2011). Briefly the current paradigm suggests that RB proteins repress E2F transcription factors until cells commit to division (i.e. the restriction point). Growth factor-induced CyclinD-Cdk4/6 phosphorylates and inactivates RB, allowing E2F-dependent transcription. The induced G1/S transcripts include Cyclin E, which initiates a positive feedback loop

enhancing its own transcription, in a manner analogous to Cln1/2 (Bertoli et al., 2013, Johnson and Skotheim, 2013). However the details of this mechanism still remain unclear, for example a recent study has demonstrated that CyclinD-Cdk4/6 is not responsible for the activating hyper-phosphorylation of RB seen at the late G1 restriction point, but instead is only responsible for mono-phosphorylation of RB earlier in G1 (Narasimha et al., 2014). Cyclin E levels peak at G1/S and are then depleted as S-phase progresses (Koff et al., 1992, Dulic et al., 1992) via Cdk2-mediated phosphorylation, which targets Cyclin E for SCF^{Fbw7}-mediated ubiquitination (Won and Reed, 1996, Hwang and Clurman, 2005). Cyclin A mRNA is an E2F-regulated transcript, and as such, Cyclin A protein levels starting to rise in S-phase and peaking in G2 (Bertoli et al., 2013, Pines and Hunter, 1991).

Cyclin B levels rise during late S and G2 downstream of numerous transcription factors (including E2F, NF-Y, FoxM1, and B-Myb) that are thought to be activated by CyclinA-Cdk2 (Fung and Poon, 2005). Once CDK becomes fully activated in mitosis it phosphorylates and activates the APC/C, which in turn ubiquitylates Cyclin A and Cyclin B targeting them for proteolysis (Pines, 2011). In *Xenopus* extracts APC/C activation is highly ultrasensitive to CDK activity and occurs on a ~15min time-delay which ensures that the negative feedback between CDK and APC/C drives robust alternating oscillations in CDK and APC/C activity (Yang and Ferrell, 2013). *In vivo* the spindle assembly checkpoint (SAC) inactivates the APC/C, delaying Cyclin B degradation until all kinetochores have microtubule attachments (Pines, 2011). However, Cyclin A degradation precedes Cyclin B proteolysis and can occur in the presence of an active SAC. This difference in timing/sensitivity is because of Cks1-mediated recruitment of CyclinA-Cdk1-Cks1 to the phosphorylated APC/C in a manner analogous to Clb5 proteolysis in *S. cerevisiae* (Di Fiore and Pines, 2010).

1.1.3 CDK inhibition by stoichiometric binding inhibitors and system level feedback

Proteins that bind CDKs stoichiometrically can act to negatively regulate CDK activity. These CDK inhibitors both transduce external signals to regulate CDK

activity in response to environmental stimuli and participate in the regulatory networks that control the dynamics of CDK activation that are characterised by multiple feedback motifs (Sherr and Roberts, 1999).

S. cerevisiae has two stoichiometric CDK inhibitors; Far1, involved in the pheromone arrest, and Sic1, involved in mitotic exit and the commitment to division. Pheromone signalling results in the Fur3 (MAPK) dependent activation of Far1 as well as the transcriptional up-regulation of Far1 levels. Far1 inhibits Cln-Cdc28 to impose a cell cycle arrest in response to pheromone to allow cell mating (Elion, 2000). The inhibition of CDK by Sic1 plays two major roles during the cell cycle. Firstly it is important to maintain a window of low Clb-CDK activity during G1 to allow appropriate origin licensing (Lengronne and Schwob, 2002) and it functions in parallel with the APC/C to inactivate Clb2-Cdc28 during mitotic exit, when Sic1 mRNA increases downstream of Ace2 and Swi5 transcription factors (Knapp et al., 1996, Toyn et al., 1997).

As CDK activity decreases due to cyclin proteolysis during mitotic exit, the protein phosphatase Cdc14 dephosphorylates Swi5, promoting Sic1 expression as well as reversing CDK-mediated phosphorylation of Sic1, which in turn further inactivates CDK (Visintin et al., 1998). These feedback loops ensure mitotic exit is irreversible: if cyclin proteolysis is disrupted during mitotic exit, cells can slip back into a mitotic state through the re-accumulation of cyclin levels. Sic1 and Cdc14 ensure that mitotic exit becomes irreversible and shorten the window in which on-going cyclin degradation is required to sustain an interphase state (Lopez-Aviles et al., 2009). Unusually, the licensing factor and CDK substrate Cdc6 has been reported to have CDK inhibiting activity and cooperates with Sic1 during the inactivation of CDK activity at mitotic exit (Elsasser et al., 1996, Calzada et al., 2001). Cdc6 binds preferentially to Clbs over Clns (Elsasser et al., 1996) and Cdc6 binding to Clb2-Cdc28, enhanced by Clb2-Cdc28 phosphorylation of Cdc6, also sequesters Cdc6 away from the chromatin, providing an additional non-canonical mechanism of CDK-mediated inhibition of origin licensing (Mimura et al., 2004).

At G1/S Sic1 protein levels drop precipitously due to the activation of Cln1/2-Cdc28, which are insensitive to Sic1 inhibition and target Sic1 for phosphorylation-

dependent SCF^{Cdc4}-mediated ubiquitination (Verma et al., 1997, Nash et al., 2001). The inactivation of Sic1 is one of the few critical functions of *S. cerevisiae* G1-cyclins, as the lethality the *cln1*Δ *cln2*Δ *cln3*Δ background can be rescued by a deletion of *sic1* (Tyers, 1996). Another positive feedback loop is involved because decreasing Sic1 levels allows the activation of Clb5/6-Cdc28 that also phosphorylates Sic1, to promote Sic1 degradation, and thus CDK activation (Koivomagi et al., 2011a). Sic1 also integrates signalling pathways into the decision to divide or not, for example Sic1 phosphorylation (T173) by the stress-activated protein kinase, Hog1, which stabilises Sic1 causing a G1 delay in response to osmotic stress (Escote et al., 2004). Sic1 phosphorylation at T173 is also important during a rapamycin-dependent cell cycle arrest, suggesting that TOR signalling during adequate nutrients conditions supresses T173 phosphorylation to permit cell division (Zinzalla et al., 2007).

The only known stoichiometric CDK inhibitor in *S. pombe* is the non-essential protein Rum1, which regulates the G1/S transition. Rum1 is important for G1-arrest in response to nitrogen withdrawal but can be complemented by the *S. cerevisiae* Sic1 protein (Moreno and Nurse, 1994, Sanchez-Diaz et al., 1998). Rum1 inhibits Cig2-Cdc2 and Cdc13-Cdc2 but not Puc1-Cdc2 or Cig1-Cdc2 complexes and promotes Cdc13 degradation during G1 (Martin-Castellanos et al., 2000). Rum1 levels peak during G1 but are abruptly down-regulated at S-phase by Cig1-Cdc2 mediated Rum1 phosphorylation (Correa-Bordes et al., 1997, Benito et al., 1998). Rum1 inhibition of CDK may also be down regulated by MAP kinase phosphorylation of Rum1 (Matsuoka et al., 2002).

Stoichiometric inhibitors of CDK include two families in higher eukaryotes: (i) the INK4 family that bind to Cdk4 or Cdk6 as a heterodimer that is incompetent for Cyclin D association and (ii) Cip/Kip (p21, p27 & p57) inhibitors that can bind a broader range of CDKs and interacts with both the cyclin and CDK subunit (Sherr and Roberts, 1999). Crystal structures of CyclinA-Cdk2-p27 indicate that a single p27 molecule inhibits CDK activity by binding the cyclin and then interacting with and reorganising the N-terminal lobe of CDK, whilst also inserting into CDK ATP binding pocket, mimicking ATP to prevent ATP binding (Russo et al., 1996a). Sic1 has been proposed to inhibit CDK in a similar manner based on functional and

structural similarities with p27 (Barberis et al., 2005, Barberis, 2012). p27 participates in the system level feedback that drives human cells through G1/S. The F-box protein Skp2, which specifically recognises CDK phosphorylated p27, targets p27 for ubiquitin-mediated proteolysis (Carrano et al., 1999, Montagnoli et al., 1999). p27 degradation is limited by Skp2 and Cyclin E/A level which are both E2F-dependent transcripts, so as CDK activity rises and induces E2F transcription, Skp2 levels increase resulting in reduced p27 levels. This increases CDK activity, which in turn induces more Skp2 expression downstream of E2F as well as targeting more p27 for Skp2 mediated ubiquitination (Yung et al., 2007).

1.1.4 Spatial dynamics and regulation of CDK activity

A significant amount of our understanding of CDK regulation and CDK output has been elucidated by experiments in soluble extract systems that are unable take account of the spatially resolved structures within the cell. Differential localisation of Cyclin-CDK complexes could control their accessibility to spatially-restricted substrates or regulators in the cell (Moore, 2013).

The most spatially dynamic cyclin is Cyclin B1, which shuttles between the nucleus and cytoplasm, in interphase its nuclear export rate dramatically exceeds its nuclear import rate resulting in a net cytoplasmic localisation (Hagting et al., 1998, Toyoshima et al., 1998, Yang et al., 1998). Towards the end of G2, Cyclin B1 accumulates at the centrosome where auto-phosphorylation of Cyclin B1's N-terminus contributes to the earliest pool of active CyclinB1-Cdk1 at G2/M (Hagting et al., 1999, Jackman et al., 2003). Shortly before nuclear envelope breakdown, Cyclin B1 dramatically and abruptly relocalises to the nucleus (Pines and Hunter, 1991, Ookata et al., 1992) which involves a spatial signal amplification mechanism in which increasing CDK activity promotes Cyclin B1 nuclear localisation (Gavet and Pines, 2010a). Nuclear CyclinB1-Cdk1 is reported to further promote the nuclear translocation and activation of CyclinB1-Cdk1 via Cyclin B1 auto-phosphorylation (Santos et al., 2012). This positive feedback is aborted in Cyclin B1 molecules harboring N-terminal phospho-mutants, which also delays the timing of the nuclear re-localisation of Cyclin B1 to coincide with, not precede, NEB

(Santos et al., 2012). However this conflicts with reports that Cyclin B1 N-terminal phosphorylation is not required for nuclear import and instead the dramatic re-localisation of CyclinB1 is achieved by modulating the nuclear transport apparatus to increase Cyclin B1 import rate (Gavet and Pines, 2010a). Regardless of the exact mechanism of nuclear import control, the interphase nuclear exclusion of Cyclin B1 prevents precocious mitotic entry in higher eukaryotes, suggesting part of the role of this carefully orchestrated localisation of CyclinB1-Cdk1 functions to spatially control the co-localisation of CDK and its substrates (Moore et al., 2003). Cyclin B2 is spatially restricted to the Golgi apparatus and promotes Golgi disassembly during mitosis. Switching the localisation determinants between Cyclin B1 and Cyclin B2 is sufficient to swap their functions, arguing that there are no major differences in biochemical specificity between Cyclin B1 and Cyclin B2 other than their spatial co-localisation with sub-pools of substrates (Draviam et al., 2001). Cyclin E and A also shuttle between the cytoplasmic and nuclear compartment throughout the cell cycle but are predominately nuclear (Pines and Hunter, 1991, Ohtsubo et al., 1995) and Cyclin E localisation to the nucleus is essential for its ability to promote DNA replication (Moore et al., 2002).

S. pombe CDK, Cdc2, is imported into the nucleus in a cyclin-dependent manner throughout the cell cycle, and its nuclear levels accumulate with that of the major mitotic cyclin Cdc13 over the cell cycle. Similar to higher eukaryotes Cdc2 and Cdc13 are enriched at the SPB before mitosis and decorate the spindle from prophase until the onset of anaphase (Decottignies et al., 2001). The synthetic forced localisation of active CDK at the *S. pombe* SPB can advance mitotic entry (Grallert et al., 2013b). An appealing model suggests that CDK activity is more readily activated at the SPB from where active Cyclin-CDK then disseminates to the rest of the cell to promote mitotic commitment via the positive feedback loops described above (Pines and Hagan, 2011). This SPB-localised activation feedback loop involves Plo1 whose recruitment, by the SPB protein Cut12, promotes mitotic commitment and is able to suppress the lethality of temperature-sensitive Cdc25 alleles (Mulvihill et al., 1999, MacIver et al., 2003).

The centrosome in higher eukaryotes may serve an equivalent function to nucleate CDK activation, consistent with the observation that active Cyclin B1-Cdk1 first

appears at the centrosome (Jackman et al., 2003). Cdc25B has been reported to specifically dephosphorylate centrosomal CyclinB1-Cdk1 and as such may participate in this localised initial activation of CDK (Lindqvist et al., 2005). Consistent with the need for this CDK activity to propagate from the centrosome, the turnover of Cyclin B at the centrosome, measured by FRAP, is rapid. (Pines and Hagan, 2011). However, the localised activation of CDK at the centrosome, or its equivalent, cannot be considered a universal and essential aspect of mitotic commitment given the instances in which spindle assemble and mitotic progression can occur in the absence of a centrosome (Khodjakov et al., 2000, Basto et al., 2006).

1.2 Functional outputs of CDK via substrate phosphorylation

The understanding of how CDK coordinates DNA replication and mitosis had lagged behind the mechanistic understanding how CDK itself is regulated (Nurse, 1990), but work over the last two decades has contributed to a rapid rise in the knowledge of CDK substrates and how their phosphorylation brings about the events of cell cycle. Key examples of CDK substrates will be discussed below to highlight the different mechanisms by which CDK phosphorylation can regulate multiple cellular functions, as well as examples of CDK substrates in *S. pombe* as a pre-text for their discussion in the subsequent chapter. The functions and phosphorylation of dozens of CDK substrates, involved in DNA replication and the events of mitosis, has been characterised in budding yeast and higher eukaryotes (Enserink and Kolodner, 2010, Errico et al., 2010).

1.2.1 Control over origin licensing and the initiation of DNA synthesis

The DNA replication of large linear eukaryotic chromosomes occurs by bidirectional semi-conservative DNA synthesis, from multiple sites across the genome (replication origins). DNA replication occurs only once every cell division cycle and this is ensured via the stepwise assembly of replication factors at origins: origins are first licensing for replication in G1 before DNA synthesis is initiated at S-phase. First of all, the Origin Recognition Complex (ORC; Orc1-6) binds replication origins (Bell and Stillman, 1992) and cooperates with Cdc6 (Cdc18 in *S. pombe*) to load the replicative helicase complex Mcm2-7 onto the DNA (Coleman et al., 1996, Donovan et al., 1997). ATP hydrolysis by ORC and Cdc6 is necessary to load Mcm2-7 (Donovan et al., 1997, Bowers et al., 2004) which is assembled around double-stranded DNA as a head-to-head double hexamer (Remus et al., 2009, Evrin et al., 2009). The essential licensing factor Cdt1 also cooperates with Cdc6 to load inactive Mcm2-7 (Maiorano et al., 2000, Nishitani et al., 2000, Tanaka and Diffley, 2002).

1.2.1.1 S-phase kinases (CDK and DDK) control origin firing

CDK cooperates with DDK (Dbf4-Cdc7) to activate the replicative helicase after it is loaded onto DNA. The timing of action of CDK and DDK seems to be important, given that the most efficient DNA replication is achieved when DDK activity precedes CDK addition in *Xenopus* and yeast lysates (Heller et al., 2011, Walter, 2000). Activation of the replicative helicase involves the assembly of the CGM complex (Cdc45, GINS complex and MCM complex) at licensed origins, resulting in pre-initiation complex formation (Siddiqui et al., 2013). CDK phosphorylation of *S. cerevisiae* proteins Sld2 and Sld3 promotes binding to an N-terminal pair of BRCT repeats in Dpb11, which is thought to facilitate the recruitment and formation of the active CGM helicase complex (Masumoto et al., 2002, Tanaka et al., 2007, Zegerman and Diffley, 2007, Labib, 2010). Synthetically forcing these interactions with Dpb11 is sufficient to bypass the requirement of CDK for the initiation of DNA replication, suggesting that Sld2 and Sld3 constitute the minimal set of CDK substrates required for an origin to initiate DNA synthesis (Tanaka et al., 2007, Zegerman and Diffley, 2007). However, bypassing Sld2 and Sld3 phosphorylation does not result in a physiological S-phase progression indicating that CDK may have other, as of yet unidentified, important substrates during S-phase. The complete *in vitro* reconstitution of DNA replication by the sequential addition of 42 different proteins has recently been reported and demonstrates that CDK phosphorylation of Sld2 and Sld3 cooperatively promotes the stable association of Cdc45 and the GINs complex with a licenced origin (Yeeles et al., 2015). This system is broadly conserved in *S. pombe* (Fukuura et al., 2011) and the highly diverged human orthologs of Sld2 (ReqQL4) and Sld3 (Treslin/Ticrr) are also critical for DNA replication (Siddiqui et al., 2013).

DDK phosphorylates the N-terminal tails of multiple MCM subunits and its activity is also necessary for DNA replication *in vitro* and *in vivo* (Weinreich and Stillman, 1999, Lei et al., 1997, Heller et al., 2011). It is thought that this phosphorylation brings about activating conformational rearrangements in the helicase complex, which may serve to stabilise the interaction of the MCM complex with Cdc45 (Sheu and Stillman, 2006). A subsection of phosphorylation events in Mcm4 also serve to alleviate an intrinsic inhibitory activity in the N-terminal region of Mcm4 (Sheu and

Stillman, 2010). *In vitro*, phosphorylation of the MCM complex is the only necessary function for DDK activity in DNA replication (Yeeles et al., 2015).

1.2.1.2 CDK inhibition of origin licensing

After G1, CDK activity rises to initiate helicase activation, but can only do so at origins licenced in the preceding G1 because the CDK activity present in S/G2/M inhibits origin re-licencing. The separation of these two mutually exclusive stages of DNA replication through inverse dependencies on CDK activity cell ensures DNA replication occurs only once per cell cycle. CDK inhibits re-licensing after G1 through multiple redundant mechanisms (Nguyen et al., 2001). Reconstituted DNA synthesis is inhibited if the ORC complex is first phosphorylated by CDK (Yeeles et al., 2015) and CDK phosphorylation of *S. cerevisiae* Orc6 prevents Cdt1 recruitment to the ORC complex (Chen and Bell, 2011). *S. cerevisiae* CDK also targets MCM for nuclear export (Liku et al., 2005) and Cdc6 for SCF^{Cdc4}-mediated ubiquitin directed proteolysis in G2 (Perkins et al., 2001). In *S. pombe,* overexpression of Cdc18 (Cdc6 ortholog) can induce re-replication (Nishitani and Nurse, 1995). Cdc18 levels drop after G1/S due to CDK phosphorylation that targets Cdc18 for SCF mediated degradation (Kominami and Toda, 1997, Kominami et al., 1998, Wolf et al., 1999) and consistent with this phospho-mutants in Cdc18 have an enhanced ability to re-replicate upon overexpression (Jallepalli et al., 1997, Lopez-Girona et al., 1998). CDK dependent phosphorylation of *S. pombe* Orc2 has also been described to play a role in the inhibition of re-licencing. Phospho-mutants in Orc2 alone do not induce significant re-replication, but do enhance Cdc18 overexpression induced re-replication (Vas et al., 2001).

In higher eukaryotes CDK also phosphorylates a number of licensing factors but the full importance of these as a mechanism to inhibit re-licensing is not presently clear (Siddiqui et al., 2013). Two other major mechanisms function in parallel to CDK to contribute to the inhibition of origin licensing in eukaryotes. Firstly proteolysis of Cdt1 in *Xenopus* is mediated through the co-recruitment of Cdt1 and the Clu4 E3-ubiquitin ligase to the PCNA homo-trimer, in a replication-dependent manner (Arias and Walter, 2005, Li and Blow, 2005, Arias and Walter, 2006). This

mechanism also exists in fission yeast (Hu and Xiong, 2006, Ralph et al., 2006, Guarino et al., 2011) and overexpression of Cdt1 in *S. pombe* can drive re-replication when combined with modest Cdc18 overexpression (Gopalakrishnan et al., 2001). In higher eukaryotes, Geminin inhibits Cdt1 except in G1 when APC/C^{cdh1}-mediated proteolysis of Geminin alleviates Cdt1 inhibition (McGarry and Kirschner, 1998, Tada et al., 2001, Wohlschlegel et al., 2000).

CDK functions via multiple substrates and alongside other mechanisms to prevent re-licencing. This probably reflects the selective advantage for incredibly strong inhibition of re-replication through multiple redundant mechanisms when duplicating the large and complex eukaryotic genome (Diffley, 2011). Whilst the requirements for DNA replication are well understood, as illustrated by the complete *in vitro* reconstitution of semi-conservative DNA synthesis, the current knowledge of how CDK activity coordinates the more complex process of mitosis is still incompletely described.

1.2.2 Initiation and coordination of mitotic progression

As discussed above (section 1.1.1.4), CDK activity regulates itself through the inhibition of Wee1 and activation of Cdc25 activity to drive entry to mitosis, as well as the activation of APC/C^{Cdc20} and inactivation of APC/C^{Cdh1} to drive mitotic exit and G1/S respectively. In addition to controlling regulatory factors, CDK also phosphorylates hundreds of proteins involved in the mechanical execution of mitosis including the structural re-organisation of organelles, mitotic spindle assembly and re-modeling, chromosome bi-orientation, condensation and segregation.

1.2.2.1 Microtubule dynamics and spindle assembly

Microtubule structures are dynamically remodeled during the cell cycle (Hagan and Hyams, 1988, Kirschner and Mitchison, 1986). In fission yeast the cytoplasmic interphase microtubule array is disassembled at mitosis when a nuclear mitotic spindle forms between two separating SPBs. The spindle elongates, as two

daughter nuclei separate, to forms a post-anaphase array and once cell division is complete interphase microtubules reassemble (Hagan and Hyams, 1988). Dis1 and Alp14 (TOG orthologs) are plus-end microtubule polymerases that promote microtubule growth (Al-Bassam and Chang, 2011, Al-Bassam et al., 2012). In *S. pombe* CDK phosphorylates the NLS in Alp7 promoting its association with Cut15 (α-importin) to drive the net nuclear import of the Alp7-Alp14 dimer, which in turn contribute to proper spindle assembly during mitosis (Okada et al., 2014). CDK regulation of Dis1 is also important for appropriate spindle formation and chromosome segregation (Nakaseko et al., 2001). CDK targets Dis1 to the kinetochore during metaphase and the dephosphorylation permits a pool of Dis1 to relocalise the spindle at anaphase (Aoki et al., 2006). SPB separation in *S. cerevisiae* requires the kinesin Kip1 that is phosphorylated by Clb2-Cdk28 and CDK phosphosites in Kip1 are essential for SPB separation (Chee and Haase, 2010). In *S. cerevisiae* the Kar9 protein is important for spindle alignment and it is asymmetrically localised to the daughter SPB in a CDK-dependent manner, possibly by phosphorylation-dependent disruption of Kar9 interactions with Bim1 (EB1 ortholog) and/or Stu2 (TOG ortholog) at the mother SPB (Liakopoulos et al., 2003, Moore and Miller, 2007).

1.2.2.2 Chromosome condensation

Fission yeast Cut3 is the SMC4 condensin subunit and is necessary for chromosome condensation and subsequent chromosome segregation during mitosis (Saka et al., 1994). CDK phosphorylates a number of N-terminal sites in Cut3 including T19, which is essential for the nuclear localisation of Cut3 and the rest of the condensin complex during metaphase (Sutani et al., 1999). In humans, CDK phosphorylation of condensin subunits during mitosis, including the condensin II subunit CAP-D3, positively controls condensin supercoiling activity *in vitro* and promotes the condensation of chromosome in *vivo* (Kimura et al., 1998, Abe et al., 2011).

1.2.2.3 Microtubule-kinetochore attachment

Bi-directional chromosome capture by the spindle in mitosis is regulated by Aurora B kinase, part of the chromosome passenger complex (CPC) (Ruchaud et al., 2007). Bir1 (Survivin), a subunit of the CPC, is phosphorylated in metaphase by CDK, facilitating binding of the centromeric shugoshin protein. This directs Ark1 (Aurora B ortholog) to the kinetochore to ensure chromosome bi-orientation (Tsukahara et al., 2010). In humans another CPC subunit (Borealin) binds Shugoshin in a CDK phosphorylation-dependent manner (Tsukahara et al., 2010). Phosphorylation of Sli15 (INCENP ortholog), another CPC subunit, in *S. cerevisiae* is also involved in CPC localisation to the kinetochore at metaphase (Pereira and Schiebel, 2003). Mutation of CDK phosphorylation sites in Sli15 or the DASH complex protein Ask1, stabilise microtubules indicating that CDK regulates these proteins to increase microtubule dynamics during metaphase (Li and Elledge, 2003, Higuchi and Uhlmann, 2005).

1.2.2.4 CDK phosphorylation at the metaphase-anaphase transition

In *S. cerevisiae*, to prepare the cell for anaphase once chromosome bi-orientation is achieved, Cdc28 phosphorylates Pds1 (*S. cerevisiae* Securin) promoting association with Esp1 (*S. cerevisiae* Seperase) and Pds1 nuclear import (Agarwal and Cohen-Fix, 2002). Cdc28 also phosphorylates Pds1 near its destruction box preventing ubiquitination by the APC/C. This contributes to a positive feedback that sharpens the metaphase-anaphase transition: activation of the CDK-counteracting phosphatase Cdc14 promotes Pds1 proteolysis, alleviating Esp1, which functions to both further activate Cdc14 as well as cleave the cohesion subunit Scc1, permitting chromosome segregation (Holt et al., 2008) (Uhlmann et al., 1999). In a similar manner to Pds1 many CDK substrates are dephosphorylated to precisely regulate their activity and/or localisation to ensure the timely execution of anaphase and cytokinesis (Kuilman et al., 2015). For example Sli15 dephosphorylation by Cdc14 allows CPC relocalisation to the spindle to modulate spindle midzone assembly during anaphase (Pereira and Schiebel, 2003). As anaphase progresses the spindle elongates to further separate chromosomes (anaphase B). Ase1 (*S. pombe* PRC1 ortholog) is an antiparallel microtubule cross-linking factor that binds

the kinesin-6 motor Klp9 during anaphase B recruiting it to the spindle mid-zone, promoting spindle elongation. This recruitment is inhibited by CDK phosphorylation of Ase1 and Klp9 and reversed by Clp1 (*S. pombe* Cdc14 ortholog) during anaphase, to ensure the appropriate regulation of spindle elongation (Fu et al., 2009). Cdk1 similarly inhibits PRC1 (human Ase1 ortholog) before anaphase (Jiang et al., 1998, Mollinari et al., 2002). Cdk1 also phosphorylates the *C. elegans* centralspindlin component ZEN-4 in metaphase, reducing its affinity for microtubules and thus its motor activity. As CDK activity declines at anaphase ZEN-4 is activated and localises to the central spindle contributing to its timely assembly (Mishima et al., 2004). Clp1-mediated dephosphorylation of Nsk1 has been reported to allow its localisation to the kinetochore-SPB interphase, which helps retain SPB-kinetochore attachments during anaphase B. CDK-mediated phosphorylation early in mitosis prevents this but also promotes its release from the nucleolus (Buttrick et al., 2011, Chen et al., 2011).

1.2.2.5 Regulation of nuclear remodeling at mitosis

CDK also regulates nuclear expansion, which accompanies the closed mitosis in *S. cerevisiae*, via the phosphorylation of Lipin (Ned1), a phosphatidate phosphatase. During interphase Nem1–Spo7 phosphatase complex antagonises Lipin phosphorylation to prevent precocious expansion of the nucleus (Santos-Rosa et al., 2005). Higher eukaryotes undergo full NEBD during mitosis, exposing chromatin to the full complement of cytoplasmic proteins (Kutay and Hetzer, 2008). The closed mitosis of many fungi may explain why they, but not higher eukaryotes, regulate the nuclear trafficking of multiple mitotic proteins by CDK phosphorylation. CDK phosphorylation also contributes to the processes of NEBD by phosphorylating Lamin proteins, promoting disassembly of the karyoskeletal lamina network (Peter et al., 1990, Heald and McKeon, 1990). Cytoplasmic Vimentin (another intermediate filament) is also disassembled by CDK phosphorylation at mitosis (Chou et al., 1990). Organelles besides the nucleus are also re-organised during mitosis probably to ensure an even distributive division of the organelle components. For example, Golgi cisternae are fragmented during mitosis due to CDK phosphorylation of the Golgi membrane receptor GM130, which disrupts the

recruitment of the vesicle-docking factor p115 (Lowe et al., 1998). The nucleolus is also disassembled in mitosis, and the inhibition of CDK activity during mitosis has been reported to increase rDNA transcription and prevent full disassembly of some nucleolar proteins (Sirri et al., 2002). In fission yeast other CDK substrates reported in the literature involved in mitosis include Mde4, Rap1 and Hcn1 (Choi et al., 2009, Fujita et al., 2012, Yoon et al., 2006).

1.2.3 Multisite phosphorylation of CDK substrates

The examples of CDK-regulated processes at S-phase and mitosis illustrate that CDK phosphorylation functions to positively and negatively regulate different aspects of different substrates, including enzymatic activity, proteolysis, subcellular localisation, multi-protein complex formation etc. Many CDK substrates are phosphorylated at multiple resides that often cluster into disordered regions of proteins (Holt et al., 2009). However, the logic describing how a CDK substrate becomes net phosphorylated is not always straight-forward.

Multisite phosphorylation can proceed via a precise logic that has been proposed to function as a 'signal processor' for CDK activity, involving the highly conserved phosphoadaptor Cks proteins. The first Cks protein identified was *S. pombe* Suc1, a multi-copy suppressor of a temperature-sensitive *cdc2* allele (Hayles et al., 1986). Suc1 orthologs in *S. cerevisiae* (Cks1) and humans (CksHs1 & CksHs2) were first identified as proteins that bind CDK (Hadwiger et al., 1989, Draetta et al., 1987, Richardson et al., 1990). The ability of Cks proteins from different species to complement *S. cerevisiae* Cks1 and to bind Cyclin-CDK complexes from other species illustrates the high level of functional conservation within the Cks protein family (Richardson et al., 1990). Cks1 is essential for S-phase and mitosis in *S. cerevisiae* (Tang and Reed, 1993) and ~50% of Cyclin-CDK complexes bind Cks1 with 1:1 stoichiometry *in vivo* (Kito et al., 2008). Cks1 functions as a phospho-adaptor by binding phospho-threonine residues and promotes multisite phosphorylation of a number of CDK substrates (Patra et al., 1999, Rudner and Murray, 2000, Koivomagi et al., 2011a, Koivomagi et al., 2013). Consistent with this model, Cks1 mutants that arrest the cell cycle do not diminish intrinsic CDK activity

towards the model substrate H1 (Tang and Reed, 1993). After binding a CDK phosphorylated Threonine, Cks1 promotes phosphorylation of secondary sites in a N-to-C terminal direction that is optimal when downstream sites are 12-16 residues C-terminal to the priming site. This suggests Cks1 positions secondary phosphorylation sites, C-terminal to the bound primer phospho-threonine, to increase their phosphorylation efficiency (Koivomagi et al., 2013). Consistent with this, CyclinA-Cdk2-Cks1 crystal structures show that the phosphate binding site in Cks proteins is positioned on the same surface of the Cyclin-CDK-Cks1 complex as the substrate binding site (Bourne et al., 1996). *In vitro* studies of the phosphorylation of a model substrate, a Sic1 fragment with a single priming site and a single downstream site, indicates that Cks1-dependent phosphorylation occurs via a processive reaction with a ~40% probability of the second phosphate being added before dissociation of the substrate (Koivomagi et al., 2013). In the absence of such a mechanism, multisite phosphorylation would instead be simply distributive, that is to say, each site is phosphorylated independently of the phosphorylation state of other sites and this does appear to be the case for a significant number of tested substrates. Processive or semi-processive phosphorylation cascades allows the amplification of substrate phosphorylation and could be critical in ensuring abrupt net multisite phosphorylation of CDK substrates during cell cycle transitions. Cks1 may also facilitate the phosphorylation of secondary sites that otherwise would never be phosphorylated in a distributive manner due to their poor phosphorylation efficiency in the absence of Cks1.

Polo-like kinase 1 (Plk1) participates in a different type of multi-site phosphorylation cascade. Plk1 contains a C-terminal domain (polo-box domain (PBD)) within which there is a charged interface between two polo-boxes that binds phospho-serine or phospho-threonine. This recruits Plk1 to specific substrates or sub-cellular locations, as well as stimulating Plk1 kinase activity (Elia et al., 2003a, Elia et al., 2003b). CDK phosphorylation has been shown to recruit Plk1 to Vimentin, BubR1 and INCENP, resulting in downstream Plk1-mediated phosphorylation at other sites within the same protein (Yamaguchi et al., 2005, Wong and Fang, 2007, Goto et al., 2006). CDK phosphorylation also directly activates Cdc5 (Plk1) in *S. cerevisiae* (Mortensen et al., 2005).

It has also been reported that CDK phosphorylation of MCM can promote DDK phosphorylation of the same MCM molecules, including sequences where CDK phosphorylation might prime phosphorylation at a -1 Serine for DDK-mediated phosphorylation (Masai et al., 2000, Montagnoli et al., 2006, Devault et al., 2008). DDK also phosphorylates residues in acidic-rich sequences suggesting that the local charge provided by CDK-mediated phosphorylation may be what is important in promoting DDK phosphorylation on the same molecule (Labib, 2010).

Cks1, Plk1 and DDK are examples the way in which CDK can amplify and diversify its phosphorylation outputs. The full nature of the CDK phosphorylation network is still mysterious. What proportion of CDK phosphorylation is distributive or processive, and how much of it involves crosstalk with other kinases, remains unclear.

1.3 CDK-counteracting phosphatases and their regulation

The nature and regulation of CDK-counteracting phosphatases has been the subject of intense research over the past decade. Work in *S. cerevisiae*, and to a lesser extent *S. pombe*, suggests that the major CDK-counteracting phosphatase in yeasts, at least during mitotic exit, is the Proline-directed Serine/Threonine phosphatase Cdc14 (*S. pombe* Clp1) (Visintin et al., 1998, Chen et al., 2011, Gray et al., 2003). Cdc14 is initially inhibited by sequestration in the nucleolus and then the FEAR and MEN pathways act sequentially to alleviate this inactivation during mitotic progression from early anaphase onwards (Stegmeier et al., 2002, Queralt and Uhlmann, 2008). Cdc14 mutants arrest in mitosis (Culotti and Hartwell, 1971) and activation of Cdc14 is nested in a positive feedback loop with Separase activation (Holt et al., 2008). In *S. pombe,* Clp1 is released from the nucleolus early in mitosis but is only fully activated once CDK activity declines, permitting Clp1 auto-dephosphorylation and full Clp1 activation (Trautmann et al., 2001, Wolfe et al., 2006). Clp1 has also been implicated in controlling the timing of mitotic entry in *S. pombe*, probably by indirectly antagonising the dephosphorylation of Cdc2-Y15 (Trautmann et al., 2001).

Cdc14 substrate dephosphorylation follows a temporal sequence as anaphase progresses (Bouchoux and Uhlmann, 2011). The order of substrate dephosphorylation corresponds to the timing of events during mitotic exit; for example, Fin1 is dephosphorylated early and functions in anaphase spindle assembly whilst Orc6 dephosphorylation occurs later to ensure that origin re-licensing only occurs once CDK activity has dropped sufficiently. Bouchoux et al. (2011) demonstrated that these small differences in the timing of dephosphorylation occur at specific CDK:Cdc14 thresholds. Substrates have differential responsiveness to the decreasing CDK:Cdc14 ratio during mitotic exit resulting in the temporal ordering of substrate dephosphorylation and the respective mitotic exit events. These differences in responsiveness are a function of the ratio of the phosphorylation and dephosphorylation rate for any given substrate (Bouchoux and Uhlmann, 2011). The biochemical determinants for these differences in phosphorylation or dephosphorylation rates are yet to be uncovered.

The role of Cdc14 in reversing CDK-mediated phosphorylation does not seem to be conserved in higher eukaryotes (Mocciaro and Schiebel, 2010) where PP2A is thought to be the major CDK-counteracting phosphatase, which unlike the monomeric Cdc14 is a trimer of a structural (A), regulatory (B), and catalytic (C) subunit (Xu et al., 2006).

The regulation of PP2A-B55 has been shown to be crucial for the entry into and exit from mitosis in *Xenopus* extracts: PP2A-B55 depletion causes hyper-phosphorylation of CDK substrates and premature mitotic entry when CDK activity is low and then fails to subsequently exit mitosis. Conversely supplementing lysates with recombinant PP2A-B55 causes a dose-dependent delay in mitotic phosphorylation (Mochida et al., 2009). PP2A-B55 activity drops precipitously during mitosis courtesy of the CDK activation of the Greatwall kinase (Gwl), which down-regulates dephosphorylation of mitotic substrates by promoting the inactivation of PP2A-B55 (Vigneron et al., 2009, Castilho et al., 2009). The regulatory circuit was completed with the identification of the Gwl substrate (α-endosulphine/Arpp19) which when phosphorylated inhibits PP2A-B55 activity directly (Gharbi-Ayachi et al., 2010, Mochida et al., 2010). Phosphorylated α-endosulphine is also a PP2A-B55 substrate, which inhibits PP2A-B55 activity by dramatically outcompeting other PP2A-B55 substrates due to its incredibly low Km and Kcat (Williams et al., 2014). PP2A-B55 and Gwl participate in multiple arms of the auto-regulatory feedback loops that control CDK activity: as CDK activity rises Gwl activation reduces PP2A activity, slowing the dephosphorylation of Wee1 and Cdc25, facilitating CDK activation and thus further downstream PP2A-B55 inactivation (Yu et al., 2006, Zhao et al., 2008). This network architecture is called a coherent feed forward loop and contributes to the non-linear bistability of the mitotic entry network that makes the decision to enter mitosis more irreversible (Domingo-Sananes et al., 2011) and sets the threshold for CDK activity required for mitotic entry (Krasinska et al., 2011). In *S. pombe* deletion of the major PP2A catalytic subunit (*ppa2Δ)* advances cells into mitosis at a small size and is synthetically lethal with Wee1 inactivation (*wee1-50*) or a background harbouring a Cdc2-T14A, Y15F mutation, hinting at a possible role for PP2A in antagonising CDK activity in fission yeast (Kinoshita et al., 1993, Navarro and Nurse, 2012).

In mitotic *Xenopus* extracts (CSF arrested) depletion of Gwl promotes mitotic exit and CDK substrate dephosphorylation even in the absence the auto-regulatory feedback loops that act on Cdk1-Y15 (using a Cdk1AF or Wee1 depletion) indicating that the function of PP2A-B55 is not just limited to participation in the regulation of CDK activity, but more broadly in global CDK substrate dephosphorylation (Castilho et al., 2009, Vigneron et al., 2009). The existence of the Gwl/α-endosulphine pathway has also been proposed to introduce a time delay into the re-activation of PP2A at the metaphase-anaphase transition to ensure that CDK substrates, such as PRC1, are always dephosphorylated after full Separase activation (Cundell et al., 2013).

Despite the obvious significance of PP2A-B55, Gwl depletion in extracts is not sufficient to increase the dephosphorylation rate of all model CDK substrates tested (Castilho et al., 2009) and CDK inactivation can still drive mitotic exit when PP2A-B55 is depleted (Mochida et al., 2009) suggesting that multiple phosphatases co-operate at mitotic exit to reverse the extensive program of mitotic phosphorylation. PP1 is directly inhibited by CDK phosphorylation of a conserved N-terminal Threonine (Dohadwala et al., 1994, Yamano et al., 1994) and chemical inhibition or depletion of the PP1 also results in cells being unable to exit mitosis (Wu et al., 2009). Recent work in *S. pombe* has suggested that PP1 and PP2A participate in a dephosphorylation relay where PP1, activated by auto-dephosphorylation as CDK activity declines, dephosphorylates and activates PP2A-B55 & B56 as mitotic exit proceeds (Grallert et al., 2015).

The precise level of interphase PP2A activity also seems important to permit DNA synthesis in interphase: if PP2A is inactivated, DNA replication is prevented even at okadaic acid concentrations that are not permissive to mitotic entry. The mechanism underlying this inhibition of DNA replication is unclear but may relate to the possibility that PP2A dephosphorylates the substrates of other kinases, beside CDK, that inhibit DNA replication in interphase (Krasinska et al., 2011).

1.4 The differential regulation of CDK outputs

Different Cyclin-CDK complexes control a plethora of different cellular process via phospho-regulation during cell division. Both CDK activity and substrate-specificity have been implicated in how Cyclin-CDKs bring about the appropriate ordering of cell cycle events. The textbook paradigm describes a model where association of S-phase cyclins with CDK directs CDK activity specifically towards substrates whose phosphorylation initiate DNA replication. As the cycle progresses and mitotic cyclins are synthesised they associate with CDK to impart an altered substrate specificity, re-directing CDK activity towards substrates whose phosphorylation is required to re-organise the cell to execute the events of mitosis (Morgan, 2007).

1.4.1 Biochemical specificity as a mechanism to order substrate phosphorylation

In 2005 Loog and Morgan illustrated the potential global significance of substrate specificity by assaying the relative phosphorylation of substrates by Clb2-Cdc28 or Clb5-Cdc28 in budding yeast extracts. 24% of 150 CDK substrates assayed were phosphorylated more efficiently by Clb5-Cdc28 than Clb2-Cdc28, normalised to H1 kinase activity. This specificity is directed towards a number of substrates involved in DNA replication and is mediated through a docking interaction between a cyclin hydrophobic patch and RxL motif on the substrate (Loog and Morgan, 2005). This docking interaction was first identified between Cyclin A2 and its substrate p27, illustrating the evolutionary conservation or co-evolution of the use of this motif to mediate cyclin-substrate interactions (Russo et al., 1996b, Schulman et al., 1998). The RxL motif dramatically decreases the Km of a substrate for the Cyclin-CDK complex *in vitro* whilst leaving the Kcat unaltered (Takeda et al., 2001). Studies on the interaction between Cdc6 and Cyclin A2-Cdk2 have shown the RxL motif is optimally positioned, at least 15 amino acids, distal to the phosphorylation site(s) (Cheng et al., 2006).

G1 cyclin specificity is also conferred by specific motifs in G1 substrates (Koivomagi et al., 2011b) (Bhaduri and Pryciak, 2011). For example, Ste5 and Ste20 phosphorylation by Cln2-Cdc28 is dependent on a LLPP and a SLDDPIQF

motif respectively. These motifs recruit Cln2 to substrates to enhance G1/S phosphorylation and can even be switched between Ste5 and Ste20 and still maintain Cln2-specific phosphorylation (Bhaduri and Pryciak, 2011). This G1 cyclin-specific phosphorylation inhibits the pheromone response signalling during G1, antagonising mating arrest, and could be important in the phosphorylation of a wider range of proteins besides Ste5 and Ste20 (Bhaduri and Pryciak, 2011). For example, phosphorylation of Sic1 by Cln2-Cdc28 depends on a VLLPP motif and serves to prime Cks1-mediated recruitment of Cln2-Cdc28 and Clb5-cdc28 to drive processive phosphorylation of less efficiently phosphorylated sites that serve as a Sic1 phospho-degron. Multiple RxL motifs on Sic1 promote phosphorylation by Clb5-Cdc28 and are important for this processive phosphorylation reaction (Koivomagi et al., 2011a). The region that substrate-docking motifs interact with on G1 cyclins is currently unknown.

Recent studies have indicated that the relative positioning of a docking motif with respect to the phosphorylation is critical. Clb5 phosphorylation of a model Sic1 fragment is promoted by an RxL motif but only when the phosphorylation site is more than 10 amino acids C-terminal to the RxL motif. In contrast the VLLPP motif promotion of Cln2-Cdc28 dependent phosphorylation is less directionally limited but does dip as the phosphorylation site and VLLPP motif converge (Koivomagi et al., 2013). As well as imparting differential substrate specificity, different cyclins activate CDK to varying degrees. Cdc28 kinase activity incrementally increases as it associates with increasing late cyclins (Cln3 < Cln2 < Clb5 < Clb2) (Loog and Morgan, 2005, Koivomagi et al., 2011b). This has been proposed to allow the phosphorylation of mitotic substrates later in the cycle, whilst the lower activity imparted by earlier cyclins is compensated for by docking interactions to bring about phosphorylation of G1/S substrates earlier in the cycle. Clb2 and Cln2 also appear to differentially modulate the consensus site specificity of CDK to adjust specificity in a site-autonomous manner (Koivomagi et al., 2011b).

Immuno-precipitation of human Cyclin A, B and E has shown that different cyclins bind to partially overlapping pools of potential substrates *in vivo* (Pagliuca et al., 2011). A significant amount of the cyclin-specific interactions could be due to differences in biochemical affinity of a cyclin for its substrates, such as Cyclin A2

docking via the RxL motif. However, it is also likely that some specificity is due to temporal differences in the in cyclin and/or substrate expression/activation.

Disruption of substrate docking motifs or cyclin hydrophobic patches reduces the phosphorylation of model S-phase cyclin substrates *in vivo*, but does not result in the complete collapse of phosphorylation at S-phase (Loog and Morgan, 2005). For example, ~100% of the *S. cerevisiae* protein Fin1 shows a phosphorylation-dependent mobility shift after release from a G1 arrest, but 60-70% of this is maintained when the Fin1-Clb5 docking interaction is disrupted (Loog and Morgan, 2005). Furthermore Sld3, a critical CDK substrate for the initiation of S-phase (Tanaka et al., 2007, Zegerman and Diffley, 2007), is equally well phosphorylated by Clb2-Cdc28 or Clb5-Cdc28 (Loog and Morgan, 2005, Uhlmann et al., 2011). This suggests specificity could be mediated through other determinants and that the timing of substrate phosphorylation is not exclusively, or even predominantly, dictated by the biochemical affinity of different cyclins for different substrates.

1.4.2 Quantitative changes in CDK activity as a core organiser for cell cycle transitions

This picture is confounded further by the genetics, which has tested the extent to which cells can tolerate the absence of certain cyclins. Deletion of Clb5&6 results in an S-phase delay (Schwob and Nasmyth, 1993) which can be rescued by the expression of Clb2 from the Clb5 promoter, as long as Swe1 is deleted to allow the premature activation of Clb2-cdc28 (Hu and Aparicio, 2005). This is because later Cyclin-CDK complexes are more strongly inhibited by Swe1 phosphorylation than earlier Cyclin-CDK complexes (Hu and Aparicio, 2005, Keaton et al., 2007, Koivomagi et al., 2011b). So *S. cerevisiae* mitotic cyclins can order S-phase and mitosis so long as their expression and activity is wired appropriately. S-phase cyclins are not capable of compensating for the mitotic functions of Clb5&6 (Fitch et al., 1992) but the requirement for G1 cyclins, Cln1-3, can be bypassed by the ectopic expression of Clb5 or the deletion of Sic1 (Schwob and Nasmyth, 1993, Tyers, 1996). This again highlights the significant redundancy between cyclins when compensating cyclin expression/activitiy is appropriately re-wired.

In *S. pombe* Cdc13 is an essential protein and no attempt to compliment Cdc13 by re-wiring the expression of other cyclins has been reported. In the absence of Cdc13 cells arrest at G2/M having undergone S-phase in a Cig1/Cig2-dependent manner (Hayles et al., 1994) (Fisher and Nurse, 1996). Strikingly *S. pombe* is able to execute all the events of the cell cycle in the absence of G1 or S-phase cyclins and only Cdc13 is strictly necessary for cell proliferation (Fisher and Nurse, 1996, Martin-Castellanos et al., 2000). Furthermore overexpression of Cdc13 and Cdc2 in G1 cells causes precocious mitotic entry resulting in mitotic catastrophe (Hayles et al., 1994). Cells running only on Cdc13 are delayed in S-phase initiation upon release from nitrogen starvation (Fisher and Nurse, 1996) and spend significantly longer in G1 than wild type cells, a delay that is dependent on Rum1. Puc1 and Cig1 are insensitive to Rum1 inhibition and phosphorylate Rum1 more efficiently, than Cig2 and Cdc13, and thus prevent the Rum1-dependent G1 extension observed in their absence (Stern and Nurse, 1998, Martin-Castellanos et al., 2000).

The general outlook is one where mitotic cyclins, with the capacity to impart the broadest and highest activity on CDK, are the most non-redundant and able to compensate for the absence of earlier cyclins. G1/S cyclins provide a more limited activity earlier in the cell cycle, combined with docking interactions to enhance the biochemical affinity, and thus the phosphorylation rates, for specific substrates. On top of this multiple layers of differential cyclin regulation confounds the understanding of the underlying biochemical principles at the core of cell cycle organisation. The observations in fission yeast lead to the proposition of the quantitative model for cell cycle progression, by Stern and Nurse, which stated that the core cell cycle organiser is composed of rising activity of CDK, not temporal waves of changing specificity (Stern and Nurse, 1996) (Figure 1.3). In G1 when CDK is absent DNA-replication origins are licenced. As CDK reaches a low activity threshold early in the cycle this is sufficient to initiate S-phase. As CDK activity rises the re-licensing of origins is inhibited in G2 and even higher CDK activity is necessary to initiate the events of mitosis. A prediction of the quantitative model is that oscillations in CDK activity below and above the S-phase threshold should allow rounds of re-replication without intervening mitoses. Early genetic studies supported such a model, for example Cdc13 repression or Rum1 overexpression

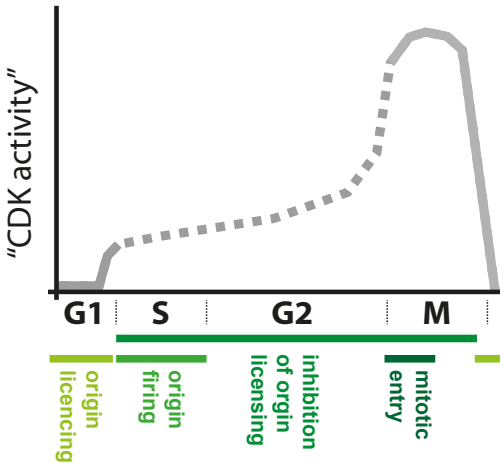

Figure 1.03 | The quantitative model for cell cycle progression CDK activity rises as cells progress across the cell cycle because of cyclin synthesis, Cdc2 dephosphorylation and Rum1 destruction. In G1 the absence of CDK activity permits origin licencing. As CDK activity rises it reaches a low level that is sufficient to initiate DNA replication, and then later a higher activity threshold is reached that permits mitotic entry.

can induce rounds of re-replication, up to ~32C DNA content (Moreno and Nurse, 1994, Hayles et al., 1994). In the case of Cdc13 repression, re-replication is dependent on Cig1 and Cig2 (Fisher and Nurse, 1996). Overexpression and then repression of Sic1 in *S. cerevisiae* can also drive a single round of origin re-licensing and then re-firing in cells previously arrested in S-phase or G2/M (Noton and Diffley, 2000).

The quantitative model has been recently tested and corroborated by simplifying the CDK network in *S. pombe* and employing chemical genetics to modulate CDK activity (Coudreuse and Nurse, 2010). Coudreuse and Nurse (2010) demonstrated that a single monomeric fusion protein between Cdc13 and Cdc2 (Cdc13-L-Cdc2) is sufficient to drive the cell cycle with near wild type physiology, in the absence of all endogenous cyclins and CDK. Specific chemical inhibition (using 1-NmPP1 and an ATP analog-sensitised Cdc2 moiety) of this Cdc13-L-Cdc2 construct showed that lower 1-NmPP1 concentrations are sufficient to inhibit mitosis than S-phase. This is also true of *S. cerevisiae* when Cdc28 is rendered specifically sensitive to 1-NmPP1 (Bishop et al., 2000). The removal and subsequent restoration of a low level of CDK activity, in a population of G2 cells, initiates a single round of DNA replication in the absence of an intervening mitosis. Similarly exposing G1-arrested cells to precociously high levels of CDK activity can drive the simultaneous initiation of S-phase and a premature mitosis. Coudreuse and Nurse (2010) were also able to drive progression through an entire cell cycle, under the control of a non-degradable Cdc13-L-Cdc2 protein, simply by sequentially chemically setting its activity at these distinct thresholds. The ability to decouple the natural order of cell

cycle progression only via the manipulation of CDK activity strongly argues that cell cycle events lack major intrinsic directionality. It also argues that the sequential passage through different activity thresholds functions as a coarse organising mechanism for cell cycle transitions, at least in fission yeast.

1.4.3 CDK and cyclin requirements and redundancies in higher eukaryotes

Various strands of evidence from work in higher eukaryotes, that have reported redundancies between the multiple CDKs and cyclins, are consistent with this model. Unlike the yeasts, higher eukaryotes have multiple CDKs that are thought to be required for distinct stages of the cell cycle: Cdk1, Cdk2, Cdk4 and Cdk6 (Malumbres and Barbacid, 2005, Satyanarayana and Kaldis, 2009). Cdk1 is essential for early embryogenesis but the combined deletion of all cell cycle CDKs in mice, besides Cdk1, permits development until mid-gestation and can give rise to mouse embryonic fibroblasts (MEFs) that can proliferate *in vitro*. In these conditions Cdk1 associates with all cell cycle cyclins but is inefficient at phosphorylating Rb causing a slowing in cell cycle time (Santamaria et al., 2007).

The redundancy between higher eukaryotic cyclins is less straightforward. In the early *Xenopus* embryos Cyclin E promotes S-phase, but Cyclin A is also capable of initiating S-phase (Strausfeld et al., 1996). Murine Cyclin E is dispensable for cell division, Cyclin E null embryos develop with surprising normality and Cyclin E genes are only necessary for specialised developmental programs involving endoreplication (Geng et al., 2003). Similarly embryos deleted for all three Cyclin D genes can give rise to proliferating fibroblasts and Cyclin D null embryos have specific developmental defects that result in embryo lethality at mid-to-late gestation (Kozar et al., 2004). Finally deletion of Cyclin A in mice is lethal early during embryogenesis but Cyclin A1 Cyclin A2 null MEFs can still be isolated and are competent for the basic process of cell division (Murphy et al., 1997, Kalaszczynska et al., 2009). In early Xenopus embryo lysates, addition of high concentrations of Cyclin A, but not Cyclin E, is also able to bring about the initiation of mitosis (Strausfeld et al., 1996) and Cyclin A2 knockdown cells delay Cyclin B1 accumulation in the nucleus and delay NEBD onset (Gong et al., 2007).

What is less clear from these studies is whether all but one higher eukaryotic cyclin is sufficient to initiate S-phase and mitosis. Cyclin A null MEFs cell division is dependent on Cyclin E, indicating that the system may not be able to be reduced further to run on only the single most essential cyclin; Cyclin B1, the deletion of which is lethal very early during embryogenesis (Brandeis et al., 1998). Consistent with this Cyclin E-Cdk1 and Cyclin A-Cdk1/2, but not CyclinB-Cdk1, can provide S-phase promoting activity in *Xenopus* extracts (Jackson et al., 1995, Strausfeld et al., 1996). The ability of Cyclin E and Cyclin A to cross compensate is consistent with the observation that there are striking structural similarities between Cyclin E-Cdk2 and Cyclin A-Cdk2 (Honda et al., 2005) and that the vast majority (81%) of putative substrates that interact with Cyclin E *in vivo* are also bound by Cyclin A (Pagliuca et al., 2011).

As described above (section 1.1.4) an important aspect of Cyclin B1 regulation is the control over its nuclear import at mitotic entry. Moore et al. (2003) re-engineered Cyclin B1, by replacing its NES-containing N-terminus region with the Cyclin E-NLS (Cyclin E/B1) or SV40 large T-antigen NLS. These constructs were able to initiate semi-conservative DNA replication after cyclin E depletion whilst wild-type Cyclin B1 could not, thus unmasking a cryptic S-phase promoting activity in Cyclin B1-CDK. However Cyclin E/B1 eventually aborts DNA replication as it also initiates mitosis (Moore et al., 2003). This inability to temporally separate S-phase and mitosis is probably in part because it transverses the two activity thresholds too rapidly, as bypassing the positive feedback loops that accelerates Cyclin B1-Cdk1 activity results in greater Cyclin B1 S-phase promoting activity.

These data are consistent with the idea that surpassing sequential CDK activity thresholds orders S-phase and mitosis in higher eukaryotes, and that a significant amount of the requirement for different cyclins is due to their differential expression and upstream regulation, not their intrinsic ability to promote specific cell cycle stages. Cyclin B1 is capable of initiating S-phase and mitosis but the use of Cyclin E or Cyclin A for the initiation of S-phase in a physiological cell cycle may allow greater temporal resolution between the attainment of the S-phase and mitotic activity thresholds and, as such, a more robust separation of S-phase and mitosis.

1.4.4 The requirement of different cyclins during developmental cell cycle programs

Clearly an important aspect to having multiple cyclins is their contribution to specific developmental situations. This could be because of two non-mutually exclusive reasons. Firstly, cyclins may target CDKs to cyclin-specific substrates, only required for certain developmental fates. Alternatively, differential developmental expression/regulation of cyclins may reduce the ability of one cyclin to compensate for another. For example despite being viable, male Cyclin A1 null mice are infertile due to failure of the first meiotic division during spermatogenesis (Liu et al., 1998) and ~50% of Cyclin E2 null male mice are infertile (Geng et al., 2003). Cyclin E is specifically important for the development of tissues that undergo endoreplication and Cyclin E embryonic lethality is due to defects in trophoectodermal endoreplicaiton and can be rescued if the extra-embryonic tissues, which includes the trophoectoderm, are wild type (Geng et al., 2003). Whilst Cyclin A1 and Cyclin A2 are dispensable for cell division, they are required for embryonic and hematopoietic stem cell division (Kalaszczynska et al., 2009).

Sexual differentiation is one of the most distinctive developmental fates and involves passage through the specialised cell division program of meiosis, which constitutes a single round of DNA replication (pre-meiotic S-phase) followed by a reductional (MI) and then equational (MII) nuclear division (Ohkura, 2015). The requirement of different kinase activities in meiosis as opposed to mitosis has been studied in more depth in the yeasts. *S. cerevisiae* Cln proteins are not required for the progression into the meiotic division cycle, which is initiated by the meiosis specific kinase Ime2, which inactivates Sic1 and inhibits APC/C^{Cdh1}. This allows the expression and activation of S-phase cyclin Clb5&6 that drive progression through pre-meiotic S-phase (Foiani et al., 1996, Dirick et al., 1998, Bolte et al., 2002, Stuart and Wittenberg, 1998, Benjamin et al., 2003). Clb1,4&5 complex with Cdc28 to initiate M1, whilst MII is orchestrated by the Clb3/5-Cdc28 complexes (Dahmann and Futcher, 1995, Carlile and Amon, 2008). These data from *S. cerevisiae* suggest that cyclin specificity may be of heightened significance during meiosis. In *S. pombe*, two additional cyclins (Crs1 and Rem1) are specifically expressed in meiosis (Malapeira et al., 2005). Cdc13 is essential for progression beyond pre-

meiotic S-phase in *S. pombe,* but five other mitotic or meiotic cyclins (Crs1, Rem1, Puc1, Cig1, Cig2) have no or very minor effects on meiotic cycle progression when individually deleted. However, Cdc13 alone is unable to drive progression through meiosis. Despite this, overexpression of the *cdc13-L-cdc2* module constructed by Coudreuse and Nurse (2010) allows progression through an azygotic meiosis in the absence of all endogenous cyclins and CDK (Gutierrez-Escribano and Nurse, 2015). This suggests that the principle of activity-dependent organisation of the mitotic cell cycle also applies to progression from pre-meiotic S-phase through MI and then MII. Minor azygotic meiotic defects and more pronounced problems during zygotic meiosis suggest that cyclin specific expression profiles or cyclin specific substrate interactions are important for the full physiology of the sexual cycle in *S. pombe* (Gutierrez-Escribano and Nurse, 2015).

This raises the question as to how CDK activity is coordinated from MI and MII without initiating an intervening round of DNA replication. Mes1 regulates the APC/C, specifically during meiosis, to prevent complete degradation of Cdc13 between the two meiotic divisions (Izawa et al., 2005, Kimata et al., 2011). This may allow CDK activity to drop low enough to permit exit from MI without dropping so low as to cause origin re-licencing. In *S. cerevisiae*, the Ime2 kinase, which phosphorylates overlapping substrates to CDK but at distinct sites (R-P-X-S/T consensus), compensates for reduced CDK phosphorylation between MI and MII to inhibit DNA replication (Holt et al., 2007). Holt et al. (2007) propose Ime2 phosphorylation is not removed between MI and MII because it is resistant to the mitotic exit phosphatase Cdc14 and that Ime2, unlike CDK, is not inactivated after MI.

1.5 Global Dynamics of CDK substrate phosphorylation in a simplified CDK network

The observations discussed above point to a model where rising CDK activity orders cell cycle events via the sequential passage through specific activity thresholds and this coarse organising mechanism is refined by multiple Cyclin-CDK complexes with alternate regulatory inputs and substrate preferences. However, the nature of the output of CDK activity at these transitions is not understood. The output of CDK, i.e. substrate phosphorylation and its dynamics, and how such dynamics are achieved are the subject matter of this thesis. To provide a global perspective, phosphoproteomics was adopted to detect and quantify thousands of phosphorylation events *in vivo*. In the second chapter I describe the definition of hundreds of CDK substrate sites in *S. pombe* and their phosphorylation dynamics during the cell cycle. Different CDK substrates are first phosphorylated at different times during the cell cycle but are only net dephosphorylated at mitotic exit. In the third chapter I present data that argues that the timing of cell cycle events is organised by differential substrate sensitivity to *in vivo* CDK activity, as well as presenting data that suggests that the progression through the G2/M transition is also ordered by differential substrate sensitivity to CDK activity thresholds. In the fourth chapter I briefly discuss the possible contribution of G1/S-phase cyclins in *S. pombe* towards CDK substrate phosphorylation. In the final results chapter, I present an analysis of the dynamics of cell cycle phosphorylation mediated by kinases other than CDK, which suggests that mitotic phosphorylation is temporally coordinated to bring about the amplification and diversification of CDK signalling.

Chapter 2. CDK dependent phosphorylation and its cell cycle dynamics

A combination of biochemical and proteomic approaches have been used over the last two decades to define the CDK consensus site and identify CDK substrates. The CDK consensus site was determined as S/T-P-X-K/R, with a minimal requirement for S/T-P (Songyang et al., 1994) and more recently a modest preference for an expanded motif has been proposed: P/C/X-X-S/T-P-X-K/R-K (Alexander et al., 2011). The strong bias for a +1 Proline is because Proline is incapable of forming an unfavourable hydrogen bond within the CDK active site, unlike all other amino acids (Brown et al., 1999). The preference for a basic residue at +3 is due a favourable ionic interaction between +3 K/R and phospho-threonine160 (in Cdk2), which helps position the S/T within the active site (Brown et al., 1999) (Holmes and Solomon, 2001). The first systematic attempt to define mitotic phosphorylation in *Xenopus* egg lysates suggested that the majority of proteins, specifically phosphorylated in mitosis, are CDK substrates (Stukenberg et al., 1997). More recently, specific covalent tagging of CDK substrates *in vitro*, and subsequent capture of phosphorylated peptides, has been used to define 68 Cdk1 substrates and 180 Cdk2 substrates in human cell lysates, many of which are also phosphorylated *in vivo* (Blethrow et al., 2008) (Chi et al., 2008). The two most extensive studies of CDK substrates have been undertaken in *S. cerevisiae*. Ubersax et al. (2003) identified 181 potential Cdc28 substrates, from a screen of 11% of the *S. cerevisiae* proteome, for proteins directly phosphorylated by Cdc28 in budding yeast cell lysates. More recently Holt et al. (2009) identified 547 CDK dependent phosphorylation events on 308 proteins, by combining *in vivo* chemical inhibition of Cdc28 with phosphoproteomics. Cdc28 substrate sites cluster in disordered regions of protein and are enriched at the consensus motif: S/T-P-X-K/R. Furthermore, a significant proportion (80/122) of substrates defined *in vitro,* that were detected as phosphorylated *in vivo* at the minimal CDK consensus site (S/T-P), are phosphorylated in a Cdc28-dependent manner *in vivo* (Holt et al., 2009).

Despite an increasing repertoire of *bona fide* and putative CDK substrates there has been no large-scale description of how CDK substrate phosphorylation

behaves during the cell cycle. Understanding how kinases, such as CDK, coordinate progression through the cell cycle requires more information than a simple list of substrates. Phosphorylation, in general, is very dynamic during the cell cycle. In the most extensive cell cycle phosphoproteomics study to date more than half of detected phosphorylation shows at least a twofold change between different cell cycle stages and the stoichiometry of phosphorylation approaches full site-occupancy at a significant proportion of phosphorylation sites during mitosis (Olsen et al., 2010). However, understanding if and how CDKs order these global phosphorylation changes *in vivo* is confounded by the presence of multiple Cyclin-CDK complexes in vertebrate systems as well as the lack of a large-scale *in vivo* definition of *bona fide* CDK substrates, outside of budding yeast.

In this chapter I describe, for the first time, the definition of hundreds of CDK substrates in *S. pombe* and the quantification of their phosphorylation dynamics during the mitotic cell cycle. SILAC was used to quantify protein levels and site resolved phosphorylation. A Shokat kinase allele (Cdc2F84G) that is rendered specifically sensitive to inhibition by bulky ATP analogs (e.g. 1-NmPP1) was used to control CDK activity *in vivo* (Coudreuse and Nurse, 2010) (Bishop et al., 2000, Dischinger et al., 2008). All experiments were performed in the simplified CDK network (*cdc13-L-cdc2 Δ2 Δ13 ΔCCP*), in which only a single Cyclin-CDK activity can direct phosphorylation and drive a physiologically normal cell cycle (Coudreuse and Nurse, 2010).

2.1 Using SILAC to analyse the phosphoproteome and proteome in *S. pombe*

Stable isotope labelling by amino acids in cell culture (SILAC) is a well-established method for comparative quantification of the proteome and involves metabolically labelling cultures with stable lysine and arginine isotopes (de Godoy et al., 2008, Ong et al., 2002, Ong and Mann, 2005). Protein samples from differentially labelled cultures are combined, digested into peptides and analysed by tandem mass spectrometry (LC-MS/MS). A given peptide from cultures labelled with heavy or light (i.e. unlabelled) amino acid isotopes will give the same spectra, with the exception that the heavy labelled peptide has a conserved six Dalton shift in its mass-to-charge (m/z) ratio (Figure 2.01a). The ratio of the heavy versus light peak intensity is used to quantify, for any given peptide, the difference in abundance between the heavy and light labelled samples. To metabolically label fission yeast lysine and arginine synthesis was disrupted (*arg1-230, lys3-37*). In *S. pombe* arginine is readily converted to other amino acids especially proline, confounding SILAC analysis, but this can be prevented by disrupting Arginine catabolism (*car2*Δ) (Bicho et al., 2010). As such all metabolic labelling of cultures was conducted in an *arg1-230 lys3-37 car2*Δ background. The linearity and dynamic range of this quantification was tested by constructing a dilution series between a heavy (H) and a light (L) protein extract and plotting the theoretical H:L ratio against the observed H:L. Figure 2.01b shows that the relationship between the observed and theoretical ratio is linear within an approximately eightfold range in either direction. Phospho-enrichment of peptides followed by LC-MS/MS allows large-scale quantification of the phosphoproteome (Holt et al., 2009, Olsen et al., 2006, Olsen et al., 2010, Sharma et al., 2014). It is worth noting a few advantages of using a SILAC-phosphoproteomics platform to study phosphorylation: i) detection of site resolved phosphorylation events, ii) detection of thousands of phosphorylation events simultaneously in the same samples, and iii) quantitative measurement of the relative differences in phosphorylation level.

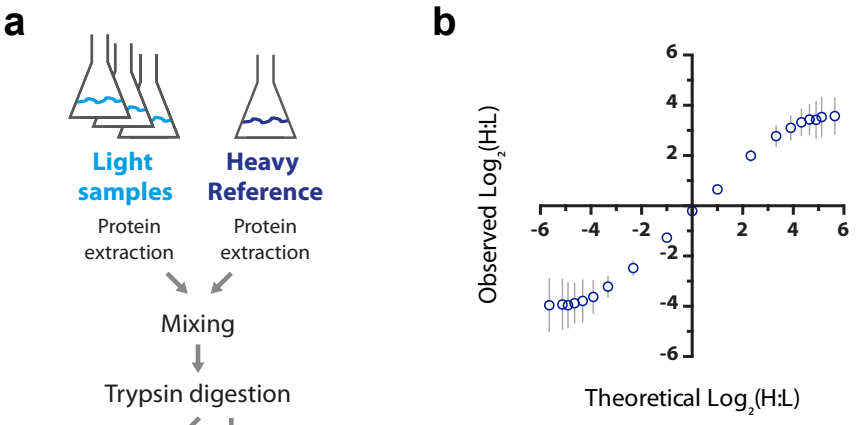

Figure 2.01 | SILAC provides linear and quantitative measurements
a Schematic of SILAC workflow to quantify relative site phosphorylation or protein levels. m/z = mass/charge.
b Plot of theoretical versus observed mean protein H:L ratios of proteins (527) quantified in all 19 mixes of protein samples from labelled (heavy) and unlabelled (light) cultures. Theoretical H:L ratios span a >2500-fold range. Error bars = standard deviation.

2.2 CDK-dependent phosphorylation

To define CDK substrates throughout the cell cycle, a heavy labelled culture was released from a G2 arrest and CDK was inactivated in mitosis (i.e. 10 minutes after release from G2 arrest) or S-phase (i.e. 65 minutes after release from G2 arrest) by the addition of 10 μM 1-NmPP1 (Figure 2.02a). The timing of S-phase and mitosis were determined by FACS (Figure 2.02b&f-h) and binucleation index respectively (Figure 2.02c-e). Protein extracts were recovered between 0 and 24 minutes after CDK inactivation. Each heavy sample was mixed with a common unlabelled reference sample, which was collected in the same conditions as the sample recovered 0 minutes from CDK inactivation in mitosis (this time point is named M00 minutes) (figure 2.02a). The unlabelled M00 min sample was used as a reference as it had the largest amount of detected phosphorylation and as such allows the detection of the most number of phosphorylation sites.

2.2.1 Global dephosphorylation in response to CDK inactivation

Both direct CDK substrates and downstream phosphorylation events dependent on CDK should eventually become dephosphorylated after CDK inactivation. Figure 2.03a shows a cumulative frequency plot of the relative changes in all detected phosphorylation after CDK inactivation in mitosis and illustrates that global phosphorylation progressively decreases as the time after CDK inactivation increases. The smaller differences between 12 and 24 min suggest that most CDK dependent phosphorylation approaches a plateau by 24 minutes. A large cross-section of the phosphoproteome is dependent on CDK activity: by 12 or 24 minutes a significant proportion of the phosphoproteome is at least twofold less phosphorylated (13.2% and 16.9% respectively) (Figure 2.03c&d), despite only a tiny fraction of the proteome having decreased similarly (1.25% and 0.78% respectively) (Figure 2.03e&f). This compares to 0.94% of the phosphoproteome and 0.53% of the proteome behaving similar, before CDK inactivation (M00 min). M00 min is a null distribution (i.e. heavy and light samples should be identical and

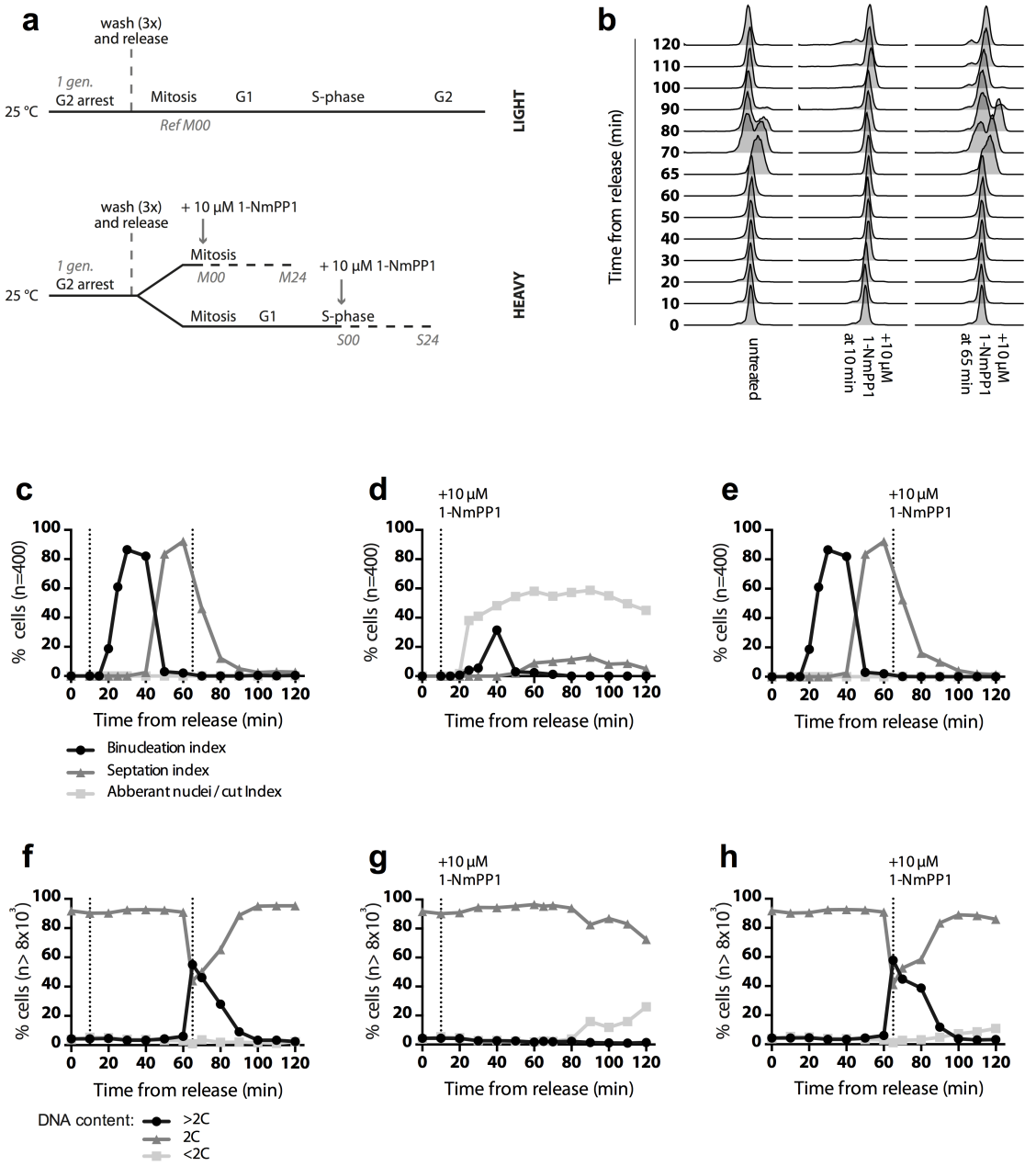

Figure 2.02 | CDK inactivation in mitosis and S-phase
a Schematic of experimental design: a labelled (H) culture (MS230) was arrested in G2 and released into mitosis, when the culture was split into two. One was treated with 10 µM 1-NmPP1 in mitosis (10 min after release from G2 arrest) and the other was untreated and split (60 min after release from G2 arrest) again were derived cultures treated with 10 µM 1-NmPP1 in S-phase (65 min after release from G2 arrest) or left untreated. Protein samples were recovered at 0, 1, 3, 6, 9, 12 and 24 min after addition of 10 µM 1-NmPP1. Protein samples from all 14 time points were mixed with a common unlabelled reference (L) (MS230, 10 min after release from a G2 arrest).
b & f-h, DNA content profiles determined by FACS and quantification. Peak in > 2C DNA content corresponds to S-phase (65-80 min).
c-e Chromosome and cell division quantified from DAPI and calcofluor stained cells. Treatment with 10 µM 1-NmPP1 in mitosis results in aberrant mitotic divisions and cut cells.
c-h Dashed lines correspond to 10 and 65 min after wash and release.

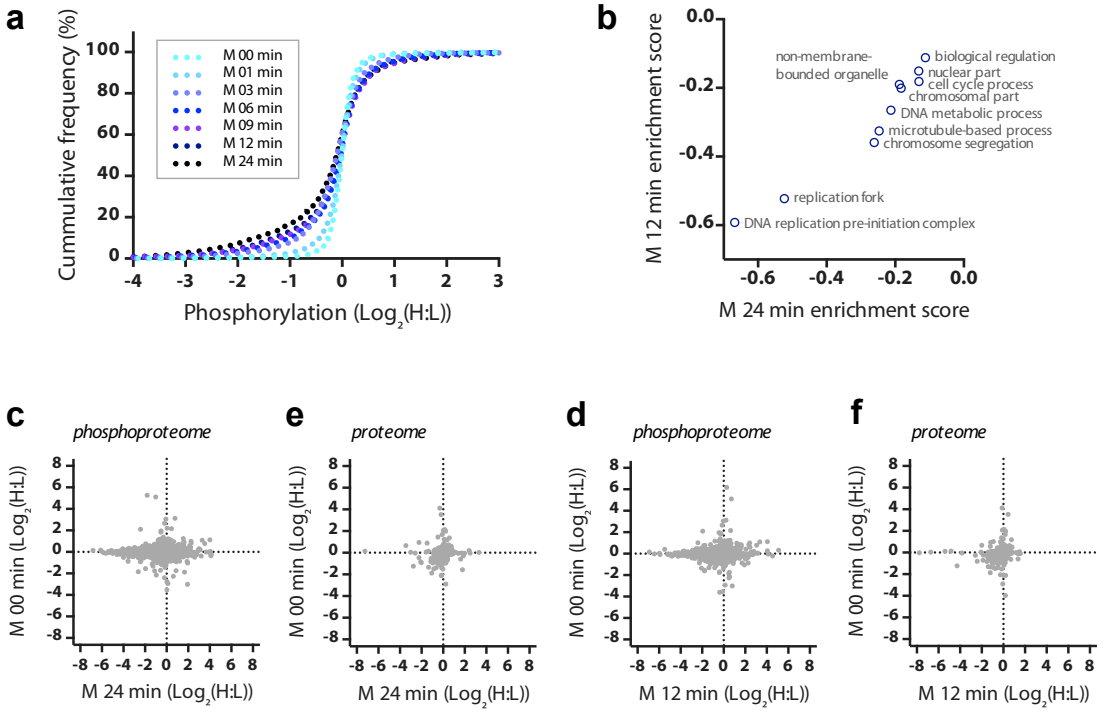

Figure 2.03 | CDK-dependent phosphorylation
a Cumulative frequency plot of the H:L ratio of all phosphorylation sites detected (loc. prob. > 0.9) at each time point after CDK inactivation in mitosis.
b 2D annotation enrichment, of gene ontology terms, comparing time points 12 min and 24 min after CDK inactivation in mitosis. Significantly depleted terms are shown.
c-f Scatter plot of before (0 min) against after (12 min or 24 min) CDK inactivation in mitosis for **c&d** phosphorylation of all detected phosphosites (loc. prob. > 0.9) (**c:** n=3397, **d:** n=3400) or **e&f** protein levels (**e:** n=3140, **f:** n=3158).

therefore theoretical H:L=1, Log_2(H:L)=0) and as such provides a good approximation of the inherent technical noise in the data. The fact that less than 1% falls outside a two fold change from the expected value of H:L=1 means the vast majority of changes in phosphorylation observed must be due to changes in the site phosphorylation occupancy, not technical noise or an indirect artefact of changes in protein level. Such a large proportion of the phosphoproteome being dependent on CDK is consistent with previous work in budding yeast (Holt et al., 2009). A 2D annotation enrichment analysis of gene ontology categories was used to analyse the entire detected phosphoproteome at 12 and 24 minutes after CDK inactivation. 2D annotation enrichment is a rank based statistical analysis that quantifies enrichment score: the more negative a categories enrichment score the greater the reduction in phosphorylation at sites annotated to a given category, in that sample (Cox and Mann, 2012). Figure 2.03b shows a strong quantitative relationship between the term enrichment scores for these two samples and

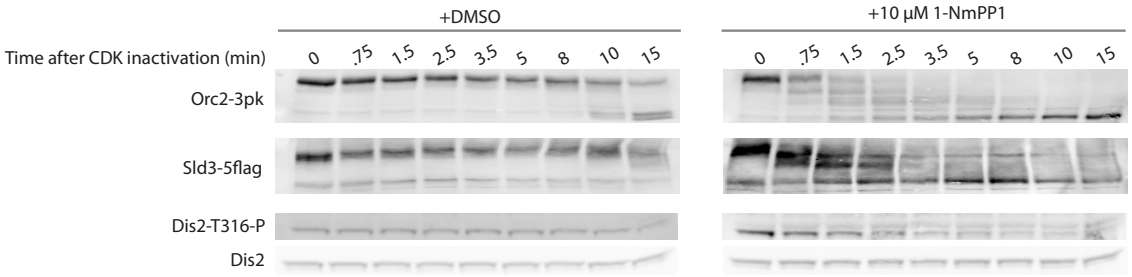

Figure 2.04 | Candidate substrate dephosphorylation during mitosis
Western blot analysis of CDK substrate (Orc2, Sld3 and Dis2) phosphorylation in mitosis.
A culture (MS212) was synchronised in mitosis and split. Each culture was treated (10 min after G2 release) with either (i) DMSO or (ii) 10 µM 1-NmPP1 (as described in Figure2.02a, lower panel). Samples were taken between 0 and 15 min after 1-NmPP1 or DMSO addition. CDK substrates are phosphorylated in mitosis and start to become dephosphorylated between 10 and 15 min without 1-NmPP1 treatment.

includes cell cycle related process. This indicates that the same classes of proteins continue to be dephosphorylated during the time course, after CDK inactivation.

2.2.2 Site resolved CDK substrate phosphorylation

Unlike previous studies, time-resolved dephosphorylation kinetics after kinase inactivation was quantified, and this was used to define CDK substrates (Figure 2.05a). Briefly, sites that fitted an exponential decay, occurred at a minimal CDK consensus site (S/T-P) and decreased at least twofold by 12 minutes after CDK inactivation, were defined as CDK substrates (see Material & Methods for details). 12 minutes after CDK inactivation was chosen because in the absence of 1-NmPP1 treatment, DNA segregation initiates and candidate CDK substrates (previously identified in the literature) start to be dephosphorylated between 10-15 min (Figure 2.02c & 2.04). Phosphorylation sites whose ratios deviated more than twofold from the expected H:L=1 at 0 minutes from CDK inactivation in mitosis (M00 min) were disregarded. The time point before CDK inactivation in S-phase (S00 min) is not a null distribution and so phosphorylation events that were only identified in the reference (unlabelled) and not the S-phase sample (S00 min, heavy) were disregarded. Using these combined criteria, 275 phosphorylation events were defined as CDK substrate sites.

Numerous properties of these sites corroborate the conclusion that they are direct CDK substrates. Firstly, they are significantly enriched on proteins annotated to

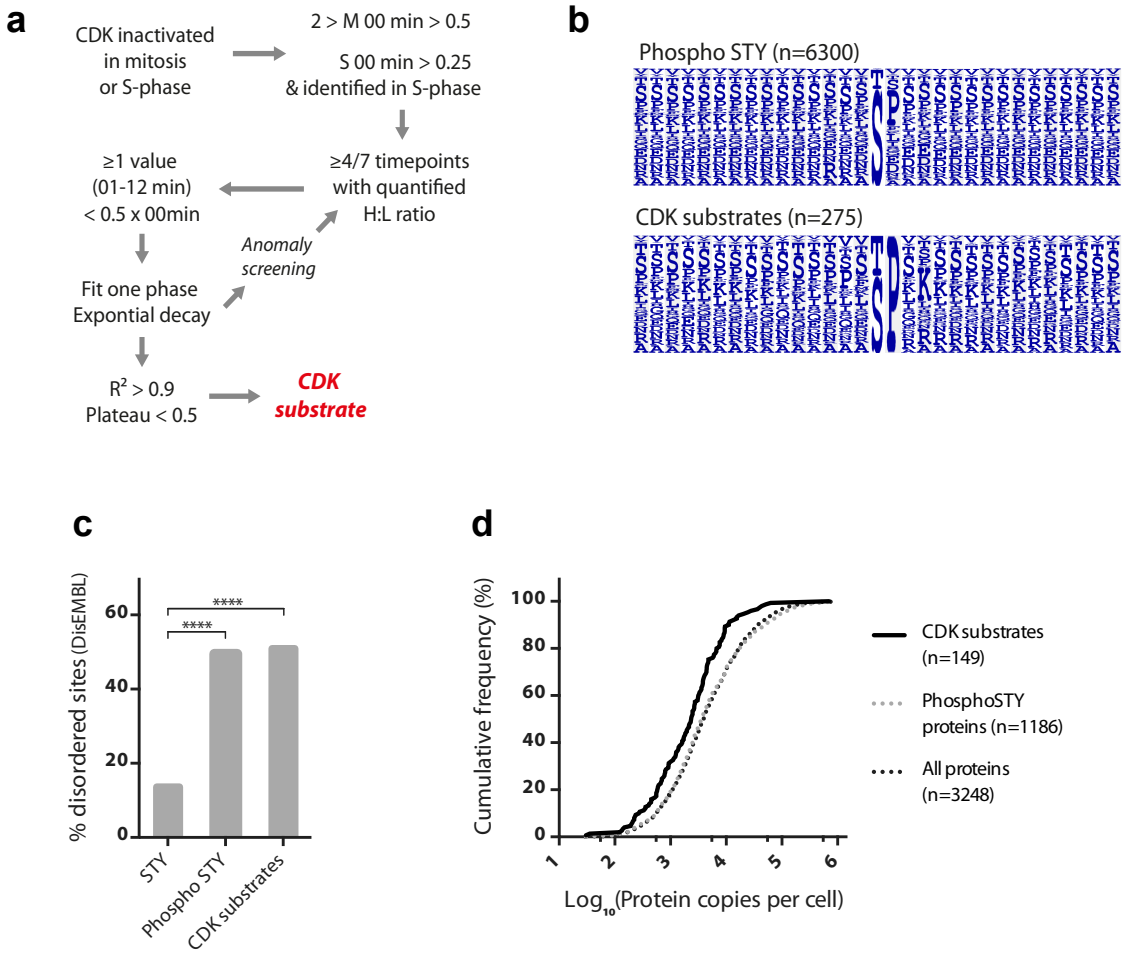

Figure 2.05 | CDK substrates and their properties
a Workflow used to to define CDK substrates (see Materials and methods section 7.4.1)
b Consensus sequences surrounding phosphorylation events (phospho STY) (loc. prob. > 0.9) and CDK substrate sites.
c Proportion of sites with predicted disordered secondary structure for STY (all potential phosphorylation sites n=437752), Phospho STY (all detected phosphorylation events, n=11407) and CDK substrate sites (n =275). Phospho STY and CDK substrates were compared to STY using a one tailed binomial test (p <0.0001) (see Materials and methods).
d Cumulative frequency of protein copies per cell (Marguerat et al., 2012) for all proteins, Phospho STY (all detected phosphorylated proteins) and CDK substrates. CDK substrates are lower in abundance than all detected phosphorylated proteins (two-tailed student t-test, p = 0.0348) (see Materials and methods).

a number of cell cycle related gene ontology terms and are enriched on orthologs of CDK substrates defined in *S. cerevisiae* (Table 2-1) (Sheet02) (Holt et al., 2009). Secondly, the majority of *S. pombe* CDK substrates reported in the literature to date are identified as CDK substrates here: Orc2, Sld3, Drc1, Cut3, Bir1, Nsk1, Alp7, Dis1, Klp9, Alp14, Ase1, Cdc25, Clp1 and Mde4. Thirdly, arginine and lysine are enriched at the +3 position, as has been described for CDK specificity *in vitro* and *in vivo* (Figure 2.05b) (Songyang et al., 1994, Alexander et al., 2011, Holt et al., 2009). Furthermore, phosphorylation at other kinases' substrate sites, that are expected to be downstream of CDK but not direct CDK substrates, are stable for a

Category value	Total size (PhosphoSTY)	Selection size	Category size	Intersection size	Enrichment factor	P value	Benj. Hoch. FDR
regulation of cell cycle	6300	275	782	63	1.8456	3.03E-07	1.35E-05
chromosome organization	6300	275	586	48	1.8765	5.84E-06	0.00014468
regulation of mitotic cell cycle	6300	275	596	52	1.9988	3.57E-07	1.33E-05
cell division	6300	275	339	36	2.4328	2.97E-07	1.66E-05
DNA metabolic process	6300	275	366	41	2.5663	9.43E-09	7.01E-07
kinetochore	6300	275	84	10	2.7273	0.0024022	0.017616
microtubule organizing center	6300	275	99	12	2.7769	0.00082962	0.014601
chromosome segregation	6300	275	185	24	2.972	9.56E-07	2.67E-05
nuclear pore	6300	275	99	13	3.0083	0.00024586	0.007212
DNA packaging	6300	275	83	13	3.5882	4.19E-05	0.00093444
chromosome	6300	275	31	6	4.434	0.0016181	0.012945
origin recognition complex	6300	275	11	4	8.3306	0.00086211	0.010838
condensin complex	6300	275	11	4	8.3306	0.00086211	0.012644

Annotation	Total size (S/T-P)	Selection size	Category size	Intersection size	Enrichment factor	P value	Benj. Hoch. FDR
phosphoS/T-P <0.25 (Holt et al. 2009)	1950	275	137	51	2.6397	1.18E-12	1.18E-12
phosphoS/T-P <0.5 (Holt et al. 2009)	1950	275	308	68	1.5655	1.09E-05	1.09E-05

Table 2-1 | CDK substrate enrichment analysis
CDK substrates are enriched in gene ontologies groups involved in cell cycle processes. CDK substrate phosphorylation is enriched on orthologs of proteins defined as having CDK dependent phosphorylation in *S. cerevisiae*. E.g. the annotation category phosphoS/T-P <0.25 (Holt et al. 2009) contains all S/T-P sites on orthologs of proteins that contained at least one S/TP sites that decreased at least 4x upon CDK (Cdc28) inactivation in *S. cerevisiae* (Holt et al., 2009).

period following CDK inactivation, suggesting downstream effects are unlikely to be miss-annotated as direct CDK substrates (see Chapter 5, Figure 5.04).

It is occasionally assumed in the literature that CDK substrates are low abundant proteins (Blethrow et al., 2008). To actually test this the protein copy number per cell, quantified by Marguerat et al. (2012), of CDK substrates was compared to all proteins with detected phosphorylation. CDK substrates are indeed significantly less abundant than the total pool of phosphorylated proteins, as well as the entire *S. pombe* proteome (Figure 2.05d) (Marguerat et al., 2012). Additionally, consistent with previous studies, CDK substrate sites and all detected phosphorylated sites are enriched in disordered regions of proteins when compared to all Serine, Threonine and Tyrosine residues in the fission yeast proteome (Figure 2.05c) (Iakoucheva et al., 2004).

In Figure 2.06 the dephosphorylation of individual sites is shown after CDK inactivation in mitosis (Figure 2.06a&b) and S-phase (Figure 2.06c&d). To assess the reproducibility of these measurements, phosphorylation half live values were also calculated from a repeat experiment (from a preliminary study) after CDK inactivation in mitosis. The repeat experiment only extended to 12 minutes after CDK inactivation, so both datasets have half lives calculated with values truncated

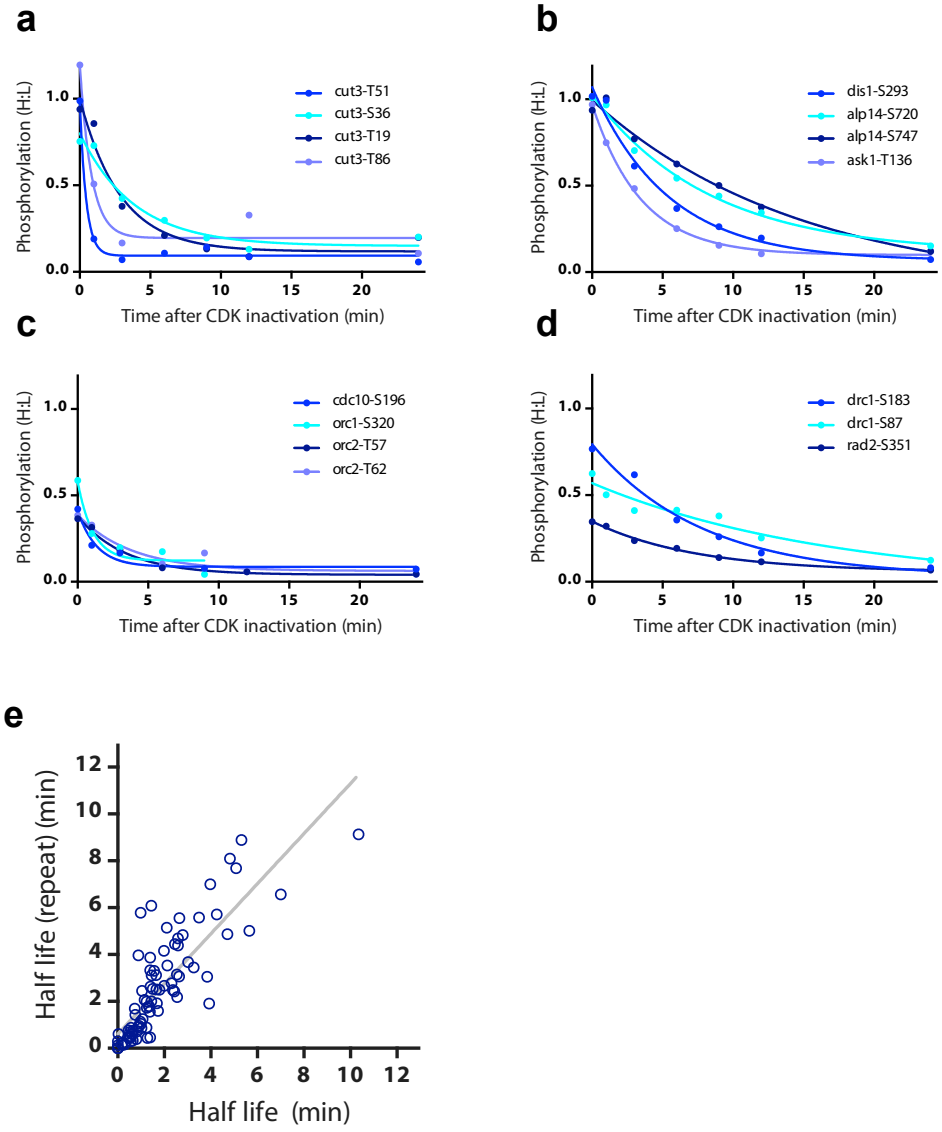

Figure 2.06 | CDK substrate dephosphorylation after CDK inactivation
a-d Examples of individual CDK substrate sites' relative phosphorylation (H:L) after CDK inactivation in **a&b** mitosis and **c&d** S-phase. Curves are a one phase exponential decay fit to the data.
e Plot of the calculated half lives against the half lives calculated from a biological repeat (MS122). Each point corresponds to a unique phosphorylation site. Biological repeat only extended to 12 min so half lives were re-calculated for both experiments truncated at 12 min. Only sites that satisfy the criteria in Figure 2.05a in both experiments and that had one phase decay fit R squared >0.95 are plotted (n=85). Linear regression line: Y = 1.069*X + 0.6062 (R squared = 0.682).

at 12 minutes. There is a strong linear correlation between biological repeats supporting the notion that this SILAC phosphoproteomics workflow generates quantitatively reproducible data (Figure 2.06e). This is further supported by the fact that there is a clear correlation between the phosphorylation half live after CDK inactivation in mitosis and the phosphorylation half live after CDK inactivation in S-phase, of the same site (see Chapter 3, Figure 3.13e). Figure 2.07a&b shows a

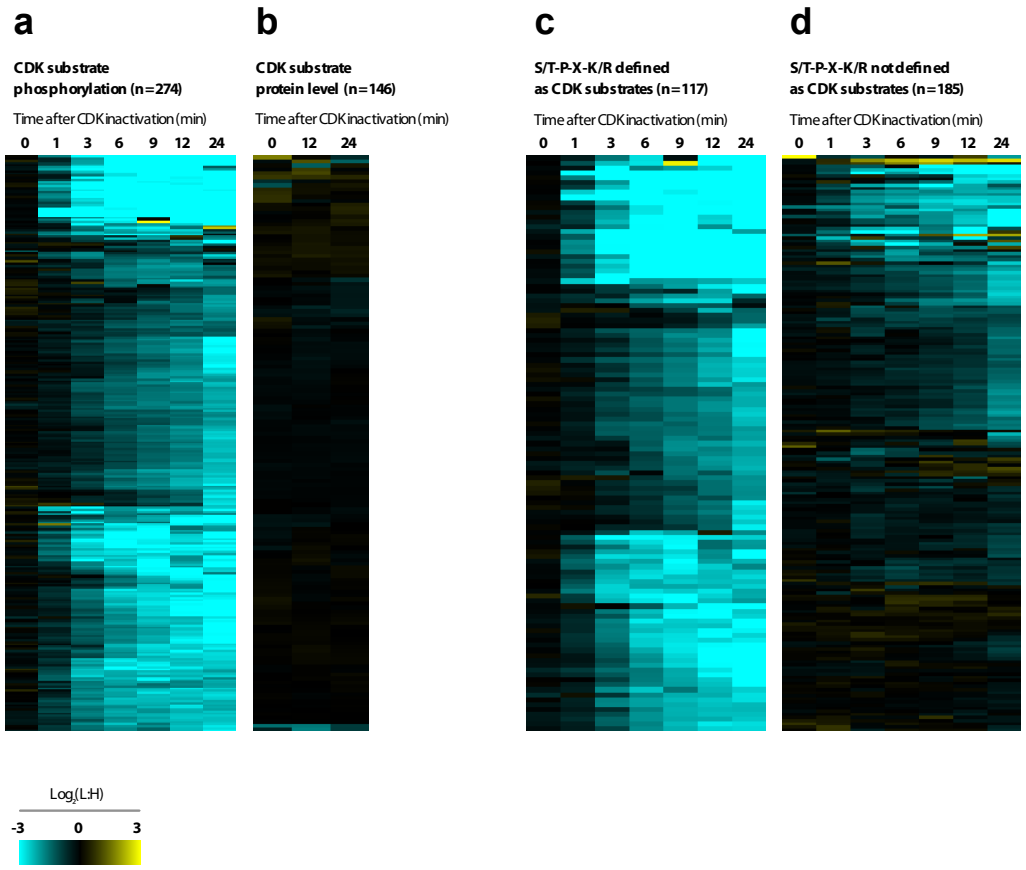

Figure 2.07 | CDK substrates and the CDK consensus site
Heat map after hierarchical clustering of **a** CDK substrate site phosphorylation, **b** CDK substrate protein levels and **c&d** all CDK consensus site phosphorylation (S/T-P-X-K/R). Each row corresponds to a single phosphorylation site and each column corresponds to a different time point after CDK inhibition. Rows are ordered by hierarchical clustering according to Euclidian distance. Values outside the display range (3 > Log_2(L:H) > -3) are set to the respective extreme.

heat map of CDK substrate site phosphorylation and protein level quantification after CDK inactivation respectively and confirms that the disappearance of detected phosphorylation on CDK substrates is due to a change in phosphorylation site stoichiometry and not an indirect effect of protein level changes. Despite the existence of a strong CDK consensus sequence (S/T-P-X-K/R), less than half (42.5%) of CDK substrate sites *in vivo* occur at this full CDK consensus site. Holt et al. (2009) found that only 29% of sites that decreased twofold after CDK inactivation are full CDK consensus sites in *S. cerevisiae*. Furthermore, only 52.6% of S/T-P-X-K/R sites had changed at least twofold by 12 minutes, similar to the 58.5% of S/T-P-X-K/R sites that decrease twofold after CDK inactivation in *S. cerevisiae* (Holt et al., 2009). Figure 2.07c shows the phosphorylation of all S/T-P-X-K/R sites defined as CDK consensus sites, and Figure 2.07d shows all S/T-P-X-K/R sites not defined as CDK substrates. Of the latter there is a significant

proportion that show no significant decrease in phosphorylation after CDK inactivation, and those that do were likely omitted as CDK substrates due to a lack of data points (before data imputation required for clustering) or anomalous decay kinetics.

2.3 Phosphorylation dynamics during the cell cycle

Characterising how CDK organises the phosphorylation of different substrates in time is critical to understanding the way in which CDK organises sequential cell

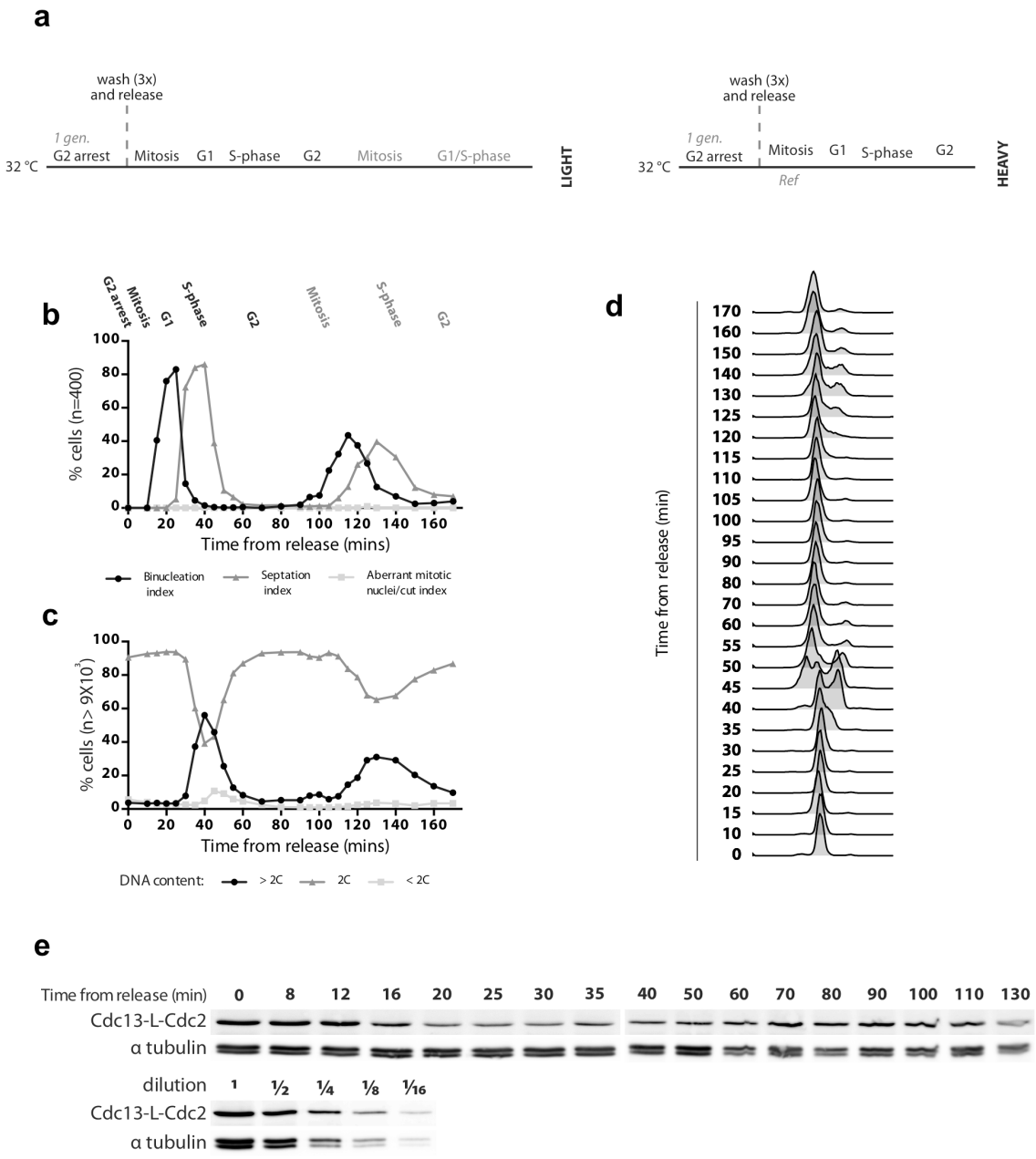

Figure 2.08 | Cell cycle synchronised culture
a Schematic of experimental design: an unlabelled (L) culture (MS230) was released from a G2 arrest and protein samples were recovered at 20 time points over the first and second cell division cycle. Protein samples were mixed with a common heavy labeled (H) reference (MS230, synchronised in mitosis).
b Chromosome and cell division quantified from DAPI and calcofluor stained cells. Corresponding cell cycle stages assigned are annotated above the graph. Peak in binucleation 20-25 min corresponds to G1 and follows mitosis.
c&d quantification and profiles of DNA content, determined by FACS, after release from G2 arrest.
e Western blot analysis of Cdc13-L-Cdc2 levels after release from G2 arrest.

cycle transitions. To describe the behaviour of phosphorylation across the cell cycle the phosphoproteome was analysed over two sequential synchronised cell division cycles, after release from a G2 arrest (Figure 2.08a). Cell cycle progression was monitored by imaging chromosome division (i.e. binucleation), cell division (Figure 2.08b) and measuring DNA content, by FACS (Figure 2.08d&c). Synchrony is high in the first cycle (>80%) but drops in the subsequent cycle (<50%). Western blot analysis shows high levels of Cdc13-L-Cdc2 protein in the G2 arrest (0 min) and first mitosis (8-16 min). Cdc13-L-Cdc2 is then degraded, coincident with chromosome division (16-20 min), and rises from G1 levels (20-30 min) through S-phase (30-50 min) and G2 until the next mitosis (90-110 min), which precedes the second of round of chromosome division (Figure 2.08e).

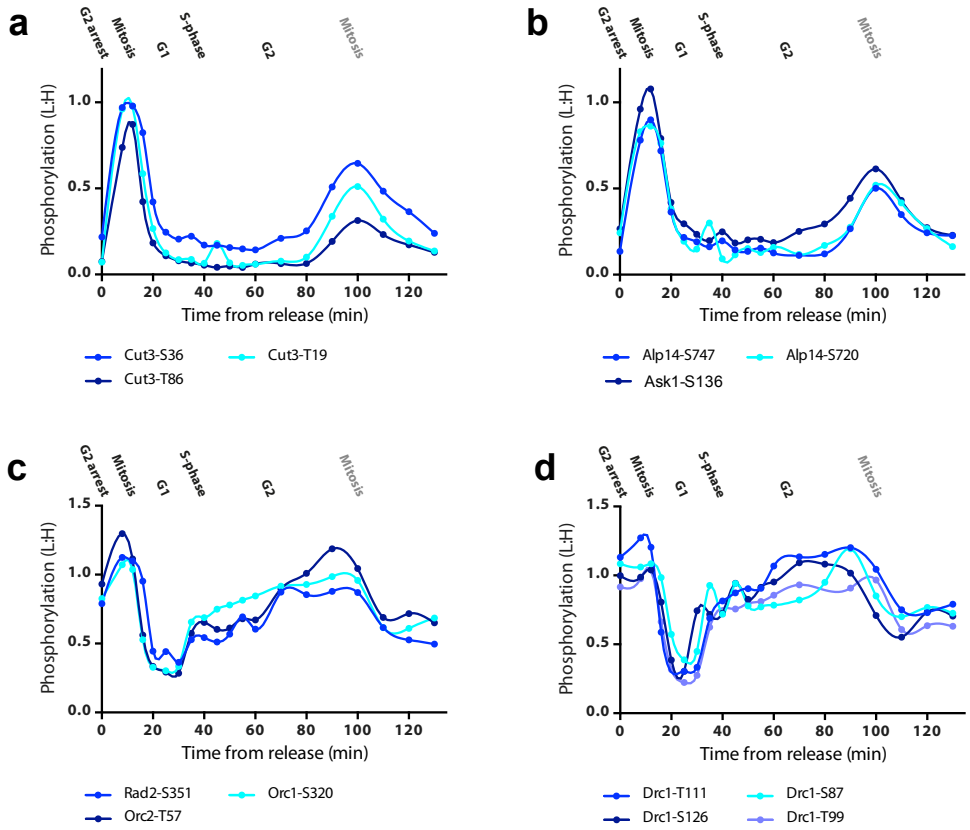

Figure 2.09 | CDK substrates have different phosphorylation dynamics during the cell cycle
a-d Examples of the relative phosphorylation (L:H) (after smoothing, see material and methods) of CDK substrate sites after release from a G2 arrest. Corresponding cell cycle stages are annotated above the graph. Spline (smoothed line) connects points.

2.3.1 CDK substrates have different phosphorylation dynamics during the cell cycle

The profiles of the relative phosphorylation, of example CDK substrate sites, are shown in Figure 2.09a-d. Substrates involved in mitotic functions (e.g. Cut3, Alp14 & Ask1) dramatically increase in phosphorylation at mitosis, after release from the G2 arrest. Phosphorylation then decreases as cells exit mitosis and only rises again as cell re-enter mitosis (90-110 min) during the second less synchronous cycle. The peak in phosphorylation at the second mitosis is half the size of the first, which is proportional to the difference in binucleation synchrony between the two mitoses. In contrast, substrates involved in S-phase (e.g. Orc2, Rad2, and Drc1) are already phosphorylated in G2 and mitosis and are then dephosphorylated during mitotic exit and G1. As cells initiate S-phase they become re-phosphorylated and their phosphorylation is maintained or increases, after S-phase completion, into the subsequent G2 and mitosis.

Figure 2.10a shows a heat map of all the CDK substrate sites detected in this experiment. Hierarchical clustering by Euclidian distance was used to identify three distinct and consistent behaviours across the time course. Firstly, the majority of sites fall into a single large cluster (black bar) and behave as, and include, mitotic proteins peaking specifically in mitosis (e.g. Cut3, Bir1, Plo1, Pic1, Dis1, Alp14 & Ask1). These 169 sites were defined as mitotic substrates. Two small clusters (light grey) show a major increase in phosphorylation at G1/S and include proteins known to be involved in DNA replication (e.g. Orc2, Orc1, Sld3 and Drc1) and, as such, were defined as S-phase phosphorylation sites (n=16). Finally, another cluster (dark grey) shows a small jump in phosphorylation at G1/S and a subsequent increase in phosphorylation at G2/M (e.g. Mis4, Rad2 and Mcm10). These substrate sites were defined as biphasic substrates (n=8). Besides these three behaviours there are a small number of sites that are broadly unchanged or that behave sporadically, likely due to technical noise, that were disregarded. The median relative phosphorylation of S-phase, biphasic and mitotic sites is plotted in Figure 2.10b, and Figure 2.10c shows the first derivative of the median relative phosphorylation with respect to time (i.e. the rate of change of phosphorylation). There is a sharp positive rate change in phosphorylation for mitotic substrate sites

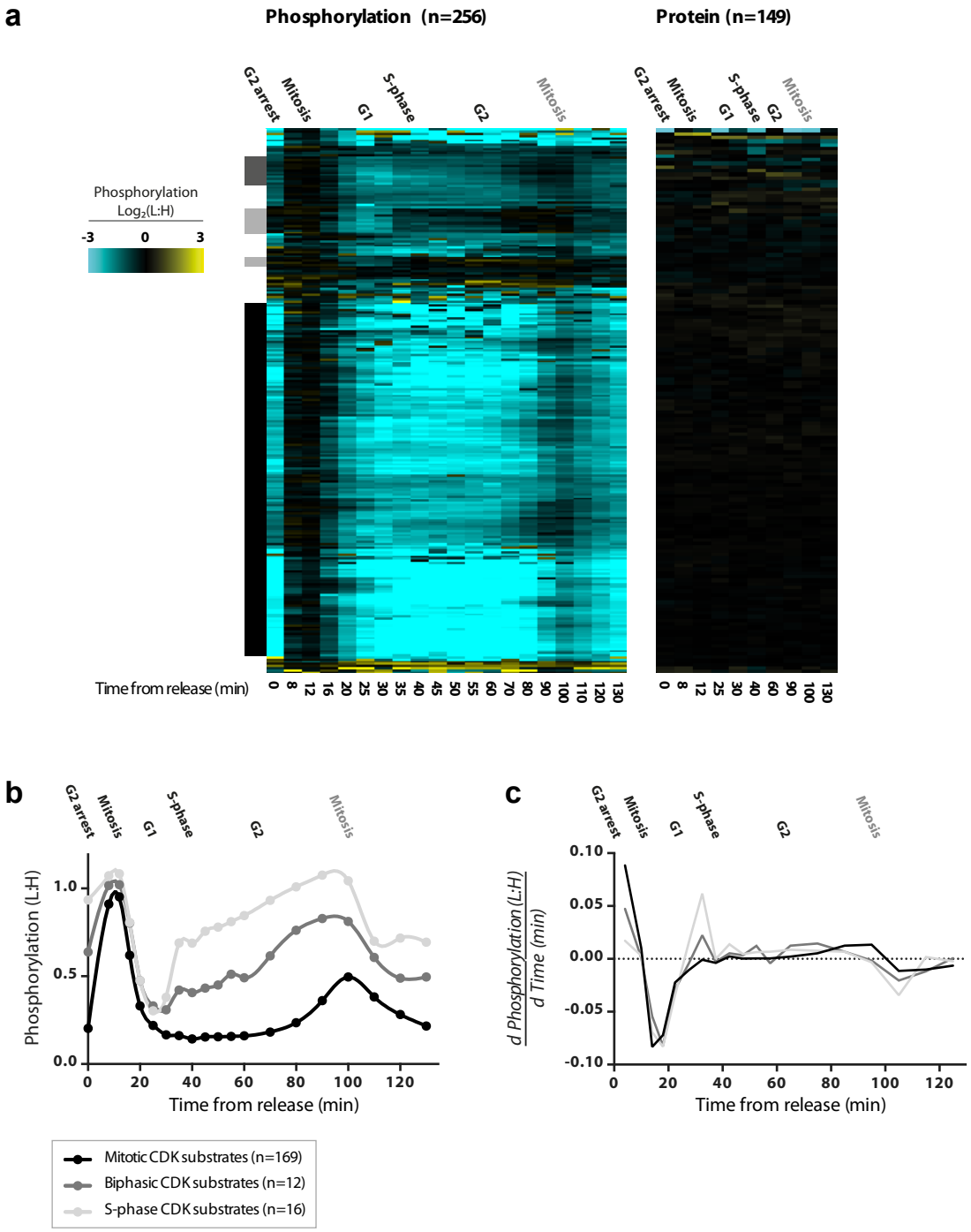

Figure 2.10 | Global dynamics of CDK substrate phosphorylation during the cell cycle
a Heat map of CDK substrate site phosphorylation and CDK substrate protein levels. Each row corresponds to a single phosphorylation site or protein and each column corresponds to a single time point after release from G2 arrest. Rows are ordered by hierarchical clustering according to Euclidian distance. Hierarchical clustering was applied after imputation to replace missing values (see method and materials). Values outside the display range ($3 > Log_2(L:H) > -3$) are set to the respective extreme.
b Median relative phosphorylation (L:H), and **c** the first derivative of median relative phosphorylation, of mitotic, biphasic and S-phase substrates after release from a G2 arrest. Corresponding cell cycle stages are annotated above the graph. Spline (smoothed line) connects points.

at G2/M, and for S-phase substrate sites at S-phase initiation. Biphasic substrates have a clear but smaller rate change at both transitions. In the second cycle biphasic substrate sites peak just before mitotic substrates, in late G2 after DNA replication is completed. All three classes show a significant negative rate change in phosphorylation during mitotic exit, i.e. rapid dephosphorylation. It is worth noting that the breadth of distribution of phosphorylation level around the second mitotic peak (~100 min) is not the same for all mitotic substrates, indicating that some substrates are phosphorylated earlier in the progression from G2 to mitosis than others (figure 2.10a). This will be discussed further in Chapter 3.

2.3.2 Changes in candidate substrate phosphorylation recapitulate global CDK phosphorylation dynamics

Four CDK substrates involved in S-phase functions (Sld3 and Orc2) or mitotic functions (Dis2 and Bir1), described in the literature, were also analysed by Western blotting to corroborate the phosphoproteomics data. Sld3, Orc2 and Bir1 phosphorylation was assayed by reduced band mobility on Phos-tag supplemented gels. Dis2 phosphorylation was detected using a site-specific anti-T316-P antibody. To monitor phosphorylation dynamics during the cell cycle, a culture was synchronised at G2/M (Figure 2.08a) and DNA content, nuclear division and cell division were monitored (Figure 2.11a&b). CDK substrate phosphorylation was assayed over the subsequent mitosis, G1, S-phase and G2 (Figure 2.11c). Total Cdc13-L-Cdc2 protein levels drop significantly at mitotic exit, followed by a gradual re-synthesis as cell progress from G1, through S-phase, to G2. Both Orc2 and Sld3 are highly phosphorylated in G2 and mitosis, become dephosphorylated in G1 and then re-phosphorylated as cells enter S-phase and their phosphorylation is maintained after the completion of S-phase. Dis2-T316 and Bir1 are specifically phosphorylated at mitotic entry and then dephosphorylated as cells progress out of mitosis. Net phosphorylation of Bir1 does fully not decrease until after mitotic exit, possibly due to Bir1 phosphorylation by other kinases. Consists with this possibility, site resolved Bir1 phosphorylation at CDK sites (S244, S278) does show a sharp and full dephosphorylation at mitotic exit. To confirm these mobility shifts (Orc2, Sld3 & Bir1) or antibody signal (Dis2-T316-P) are dependent on both

phosphorylation and CDK activity, three conditions with different CDK activity levels (high, intermediate & low) were tested and protein extracts were dephosphorylated with lambda phosphatase (Figure 2.11d). These behaviours are consistent with the phosphorylation dynamics observed for more than a hundred CDK substrates using phosphoproteomics and the conclusion that different CDK substrates are first phosphorylated at different times during the cell cycle. A limitation of phosphoproteomics is it is difficult to calculate stoichiometry of phosphorylation events. However, for the three candidate substrates where phosphorylation has been assayed by mobility band shifts (Orc2, Sld3 & Bir1) the vast majority of the protein undergoes a change in net phosphorylation at the respective cell cycle transitions.

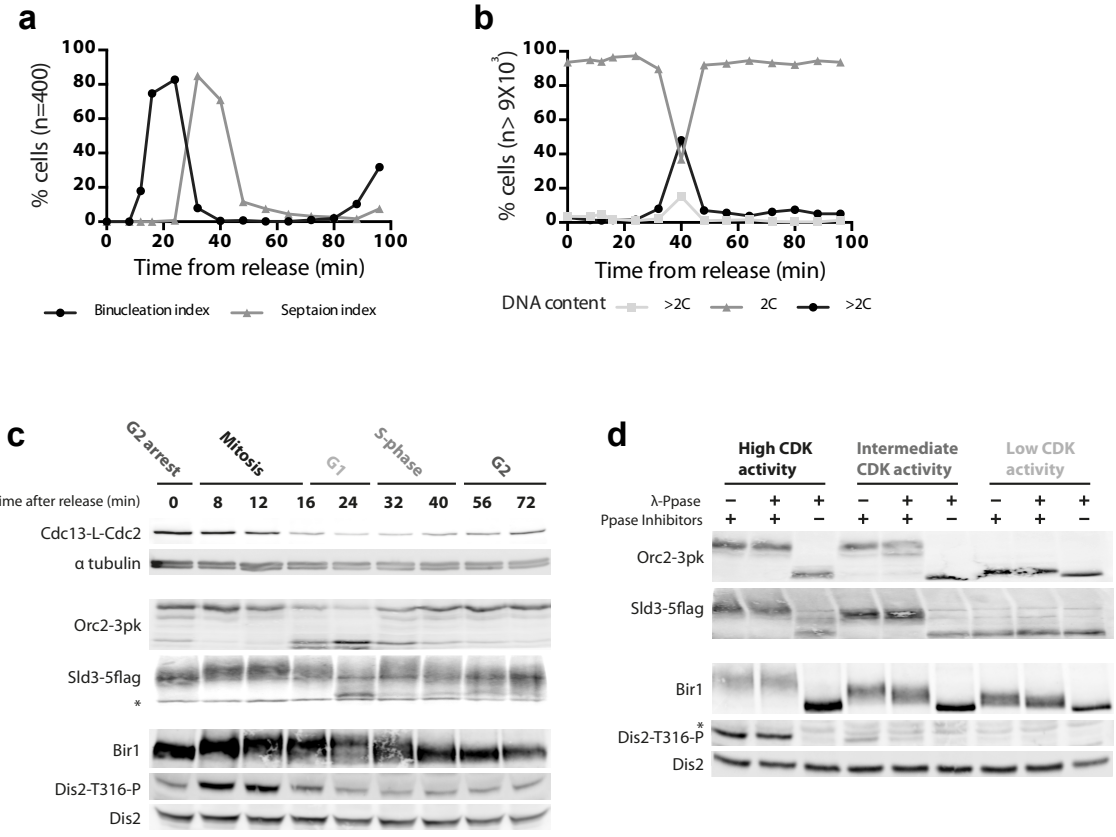

Figure 2.11 | Candidate CDK substrate phosphorylation dynamics during the cell cycle
Cells (MS132) were synchronised, as in Figure 2.08a.
a Quantification of DAPI and calcofluor stained cells after release from G2 arrest.
b Quantification of DNA content, determined by FACS.
c Western blot of Cdc13-L-Cdc2, α tubulin and four CDK substrates: Orc2, Sld3, Bir1 and Dis2 after release from a G2 arrest. Phosphorylation is monitored by mobility shift for Orc2, Sld3 and Bir1. Dis2 phosphorylation is detected directly with anit-T316-P. Asterisk (*) marks non-specific bands.
d Dis2-T316-P signal and band shifts are CDK and phosphorylation dependent. Low CDK activity = 20min 10 μM 1-NmPP1 treatment (MS132), intermediate CDK activity = G2 arrest (1 generation in 1 μM NmPP1) (MS132) and high CDK activity = mitosis (10 min after release from G2 arrest) (MS132). Each sample was treated with: (i) mock, (ii) lambda phosphates + phosphatase inhibitors or (iii) lambda phosphatase.

2.4 Discussion

The main conclusions of this chapter are as follows. Firstly, hundreds of CDK substrate sites have been defined in *S. pombe* for the first time. Secondly the cell cycle dynamics of the proteome and phosphoproteome have been analysed and indicate that different CDK substrates have dramatically different behaviours in the dynamics of their phosphorylation during the cell cycle. S-phase substrates, first phosphorylated at G1/S are not dephosphorylated after S-phase but their phosphorylation is sustained across the rest of the cycle until cyclin degradation at mitotic exit. Mitotic substrates do not show an increase in phosphorylation at G1/S but instead show a dramatic increase at G2/M. The behaviour of S-phase and mitotic substrates starts to explain how a single CDK temporally separates the initiation of S-phase and mitosis: substrates required at S-phase are first phosphorylated earlier in the cell cycle than mitotic substrates and everything is reset as the cell cycle is completed. This progresses the question from how a single CDK orders cell cycle events to how does a single CDK temporally order the initial increase in phosphorylation of different classes of CDK substrates? How CDK activity contributes to this differential behaviour is the topic of chapter 3. Finally, there is a third class of substrates whose phosphorylation changes at both the G1/S and G2/M transitions (biphasic substrates). The significance of these phosphorylation events is not clear but may have a role in the completion of S-phase or the preparation of replicated chromosomes for mitosis, given that they peak before mitotic substrates at G2/M.

The majority of previously described fission yeast CDK substrates have been identified here, often with multiple phosphorylation events detected per protein. Proteins described as important for the execution of S-phase and mitosis are phosphorylated at the respective cell cycle stages. The identification of multiple conserved and essential CDK substrates, for example the critical DNA initiation proteins Sld3 and Drc1 (Sld2 ortholog), supports the classification of other identified sites as *bona fide* CDK substrates. Furthermore, a number of novel CDK substrates in *S. pombe* are orthologs of CDK substrates described in other systems. For example, phosphorylation of multiple ORC proteins has been reported in budding yeast and higher eukaryotes and here we identify phosphorylation events

on Orc1 (S320, T292) that increase at S-phase initiation, in addition to phosphorylation in the previously described substrate Orc2. Numerous orthologs of budding yeast Cdc28 substrates are also novel fission yeast Cdc2 substrates as discussed further in Chapter 6. These examples and the fact the CDK substrates described here are enriched in orthologs of Cdc28 dependent phosphorylation described by Holt et al. (2009), suggest that numerous regulatory outputs of CDK have been conserved over, at least, the 330–420 million of years of evolution between budding and fission yeast (Sipiczki, 2000). Possible functions of as yet uncharacterised CDK regulation on certain substrates will be explored in the general discussion. Less than 10% of CDK substrates with consistent dynamics over the cell cycle are S-phase substrates, suggesting the CDK regulation of S-phase is significantly simpler than that for mitosis, consistent with the fact that the initiation of eukaryotic DNA replication, and the inhibition of licensing by CDK, can be fully reconstituted *in vitro* (Yeeles et al., 2015). It also hints that when considering the differences between mitotic and S-phase substrates, that S-phase substrates can be considered the exception to the rule and that determinants to increase S-phase substrate phosphorylation earlier in the cycle may have been easier to evolve than a mechanism to decrease the phosphorylation of ten-times more mitotic substrates.

Not all CDK substrate sites *in vivo* reside at the optimal consensus sequence (S/T-P-X-K/R). Both in fission yeast and budding yeast the majority of defined substrates are phosphorylated at only the minimal consensus site (S/T-P). Ubersax et al. (2003) also failed to detect any CDK phosphorylation on 123 proteins that contained S/T-P-X-K/R sites. This could of course be because these sites are not appropriately exposed for phosphorylation. However, Figure 2.07 shows examples of S/T-P-X-K/R site that are phosphorylated *in vivo* but are not dependent on CDK activity. Its possible that CDK is capable of phosphorylating these sites but other kinases may phosphorylate these sites with such high stoichiometry that CDK has no net contribution to phosphorylation. Alternatively, CDK may be unable to phosphorylate these sites because some other constraints or requirements are imposed on the CDK-substrate interaction.

CDK substrates are lower abundance than the average phospho-protein, consistent with the view that regulatory proteins tend to be lower abundance. The “futility” of phosphorylation-dephosphorylation cycles between a kinase and phosphatase has been discussed in terms of energy (ATP) wastage (Mochida and Hunt, 2012). At first sight the energy wastage of futile cycles on CDK substrates might seem significant given the large number CDK substrates and the rapid dephosphorylation rates they are subjected to (median half life = 2.21 min). However, this fails to consider the absolute abundance of phosphorylation sites that CDK regulates. Ultradeep phosphoproteomics analysis, which mapped >50,000 phosphorylation events in human cell lines, concluded that phosphorylation adheres to a Paerto-like principle, where 80% of total phosphorylation occurs at 11% of unique phosphosites (Sharma et al., 2014). This means the vast majority of unique phosphorylation sites (89%) in humans account for only a small fraction of cellular ATP used on protein phosphorylation reactions (20%). The CDK substrates defined here are low abundant proteins and as such the phosphorylation-dephosphorylation cycle can probably be considered minimal in terms of energy expenditure within a cell.

Chapter 3. Differential sensitivity to CDK activity orders substrate phosphorylation to temporally organise cell cycle events

The data presented in Chapter 2 demonstrates there are hundreds sites phosphorylated by CDK that, despite being phosphorylated by the same kinase, have different phosphorylation dynamics during the cell cycle. CDK activity rises during the cell cycle and is higher in mitosis than in S-phase, in both *S. pombe* (Moreno et al., 1989) and higher eukaryotes (Arion et al., 1988, Draetta and Beach, 1988, Labbe et al., 1988b). The observation that a single Cyclin-CDK complex is capable of ordering S-phase and mitosis in *S. pombe* lead to the proposal of the quantitative model for cell cycle progression; where S-phase is initiated at a low CDK activity threshold and high CDK activity is required to initiate mitotic entry (Stern and Nurse, 1996). This model has gained strong support from recent work, discussed at length in the Introduction (Coudreuse and Nurse, 2010). If S-phase substrates are more sensitive to CDK activity than mitotic substrates, rising CDK activity during the cycle could then order substrate phosphorylation to organise cell cycle events. In this chapter I will describe the quantification of *in vivo* substrate sensitivity to CDK activity and experiments to test if changes in activity-dependent substrate phosphorylation are sufficient to explain the ordering of cell cycle events.

3.1 CDK substrates phosphorylation sensitivity to CDK activity *in vivo*

To quantify the relative sensitivity of substrates to CDK activity, CDK activity was titrated *in vivo* and the phosphoproteome was assayed to monitor CDK substrate phosphorylation. A range of titres of CDK activity were achieved by arresting cells, to accumulate Cdc13-L-Cdc2, eliminating all CDK activity with 10 μM 1-NmPP1 (30 min) and then washing and releasing cells into a range of 1-NmPP1 concentrations () (Figure 3.01a). The phosphoproteome was analysed at a fixed time after release (10min) to assess CDK substrate phosphorylation across a range of CDK activities. CDK activation is highly non-linear due to positive and double negative feedback loops (Solomon et al., 1990, Pomerening et al., 2005). Bypassing the auto-regulatory feedback loops that act on the inhibitory T14/Y15 phosphorylation on CDK can linearise the CDK activation profile (Pomerening et al., 2005). As such, mutations in the Cdc2 moiety (T14A Y15F, referred to as AF, (Coudreuse and Nurse, 2010)) that bypass this feedback were used to ensure a more linear output of CDK activity in response to 1-NmPP1 concentrations. Protein samples were also extracted from cells with a wild type Cdc2 moiety at the extremes of the titration

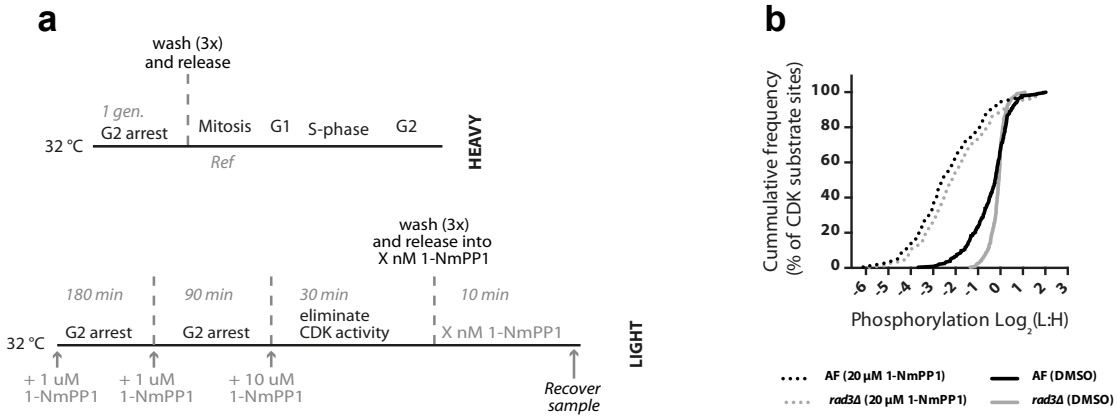

Figure 3.01 | Titration of *in vivo* CDK activity
a Schematic of experimental design: cultures were arrested for 4.5 h (3 h (1 μM 1-NmPP1) + 1.5 h (2 μM 1-NmPP1)) and then CDK activity was eliminated by the addition of 10 μM 1-NmPP1. Cultures were then washed & released into media containing 2% DMSO or 1-NmPP1 (5 nM, 15 nM, 50 nM, 150 nM, 300 nM, 1 μM, 2.5 μM, 5 μM, 7.5 μM, 10 μM, 20 μM). Protein samples were taken 10 min after the wash was started and were mixed with a common heavy labelled (H) reference (MS230, synchronised in mitosis). An AF strain (MS86: *cdc13-L-cdc2AF Δ2 Δ13 ΔCCP* (T14A, Y15F mutations in cdc2 moiety)) was used to bypass positive feedback on CDK activity. Samples were also recovered from a *rad3Δ* (MS87: *cdc13-L-cdc2 rad3Δ Δ2 Δ13 ΔCCP*) in DMSO and 20 μM 1-NmPP1.
b Cumulative frequency of CDK substrate phosphorylation (L:H) in *rad3Δ* and AF after release into DMSO or 20 μM 1-NmPP1. AF (DMSO): n=236, AF (20 μM 1-NmPP1): n=218, *rad3Δ* (DMSO): n=242, *rad3Δ* (20 μM 1-NmPP1): n=220.

series (DMSO and 20 μM 1-NmPP1). To ensure that activation of the DNA damage/replication checkpoint did not feed into CDK activity, a *rad3*Δ was introduced into this background. This was not necessary in the AF background, as checkpoint dependent inactivation of Cdc2 works through T14Y15 phosphorylation (Rhind and Russell, 1998, Coudreuse and Nurse, 2010). Figure 3.01b shows the cumulative frequency of the relative phosphorylation for all CDK substrates in DMSO or 20 μM 1-NmPP1 for the *rad3*Δ or AF strains. The vast majority of CDK substrates are dephosphorylated in 20 μM 1-NmPP1 in both backgrounds and there is a massive global increase in CDK substrate phosphorylation between 20 μM 1-NmPP1 and DMSO for both strains, despite the fact that the AF strain does not achieve the maximal phosphorylation of all substrates in DMSO that is observed in *rad3*Δ.

3.1.1 S-phase substrates are more sensitive to CDK activity than mitotic substrates

Figure 3.02a-d shows examples of the relative phosphorylation for CDK substrates. Substrates involved in S-phase (e.g. Drc1, Orc1 & Rad2) are dephosphorylated up to 7.5 μM 1-NmPP1 and become phosphorylated between 1-5 μM 1-NmPP1. In contrast CDK substrates involved in mitotic functions (e.g. Cut2, Dis1, Alp14 & Ask1) are not phosphorylated below 1 μM 1-NmPP1 and become maximally phosphorylated between 15 nM and 50 nM 1-NmPP1. The dose response to 1-NmPP1 of most CDK sites can be fitted to a four parameter sigmoid function, which was used to calculate an IC50 value (nM) of phosphorylation to 1-NmPP1 for more than a hundred CDK substrate sites. The median IC50 values are 215nM, 1726 nM and 3658 nM for mitotic, biphasic and S-phase substrates respectively. Figure 3.02e shows the cumulative frequency of IC50 values for each category of substrates. Figure 3.02f shows the cumulative frequency of the sigmoidal curve Hill slope values for each category. The Hill slope is a measure of how abrupt or steep the rise in phosphorylation is between maximal and minimal phosphorylation. The median hill slopes are -1.4, -1.3 and -3.6 for mitotic, biphasic and S-phase substrates respectively. In conclusion, mitotic CDK substrate phosphorylation is

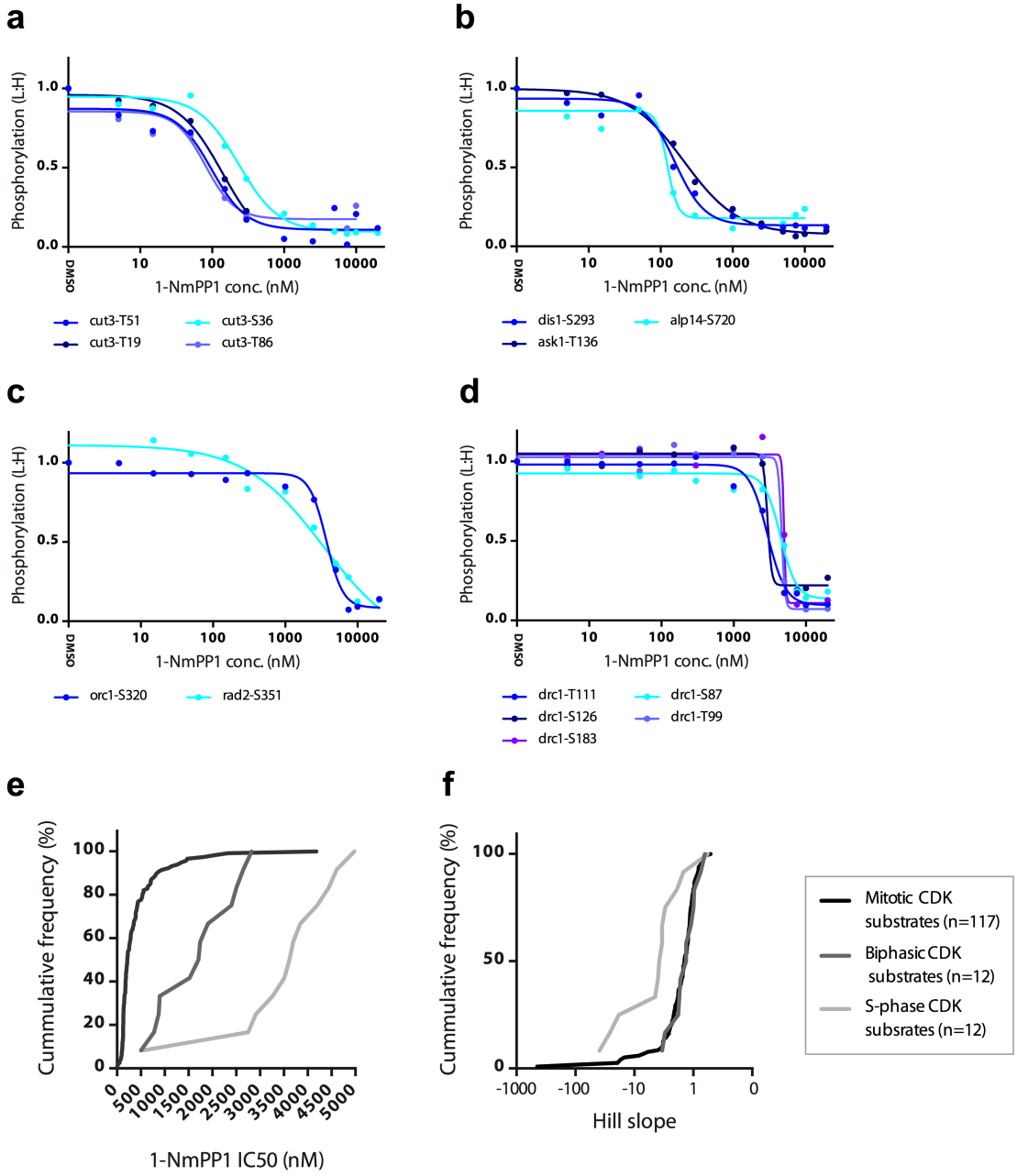

Figure 3.02 | Mitotic and S-phase CDK substrates have different sensitivities to CDK activity
a-d Examples of individual CDK substrate sites' relative phosphorylation (L:H) over a range of 1-NmPP1 concentrations. Relative phosphorylation was normalised so phosphorylation in DMSO = 1. Curves are a sigmoidal fit to the data.
e Cumulative frequency of the IC50 to 1-NmPP1 of mitotic, biphasic and S-phase CDK substrates.
f Cumulative frequency of the hill coefficient of mitotic, biphasic and S-phase CDK substrates.

more sensitive to 1-NmPP1 inhibition and conversely less sensitive to CDK activity than biphasic substrates, which are in turn more sensitive to CDK activity than S-phase substrates. S-phase substrate phosphorylation is also more ultrasensitive to CDK as the rise in S-phase phosphorylation is more step-like and less gradual than that of mitotic or biphasic substrates (i.e. more negative Hill slope).

As well the significant differences between S-phase, biphasic and mitotic CDK substrates, there are also small differences between the phosphorylation sensitivity of different mitotic substrates. Figure 3.03a shows examples of sites on three different mitotic substrates: Plo1, Pic1 and Bir1. Bir1 phosphorylation is more sensitive to CDK activity than Pic1 and Plo1, with Plo1 the least sensitive of the three. Sites within the same protein appear to behave more similarly to each other than sites between different proteins. To test if this is a global trend the IC50 was calculated for 48 CDK sites that reside on substrates with more than one mitotic CDK substrate site (n=19). The absolute difference between the IC50 of two sites (ΔIC50) was calculated for every pair of mitotic CDK sites in the same protein (intra-protein comparisons) and between all possible pairs of the 48 sites irrespective of which protein they resided in (all comparisons). Figure 3.03c is a cumulative frequency plot of these two ΔIC50 populations and shows that the differences between sites in the same protein are smaller than all differences between all sites, illustrating that sites on the same protein tend to behave similarly to each other.

3.1.2 Substrate sensitivity correlates with the timing of phosphorylation at the G2/M transition

As noted in Chapter 2 (section 2.3.1) the timing of phosphorylation during the G2/M transition is not identical for all mitotic substrates (Figure 2.10a). Figure 3.03b shows Bir1 is phosphorylated a little earlier during G2/M than Pic1, which in turn is phosphorylated before Plo1. To test if these small differences in the sensitivity to CDK are relevant to the dynamics of phosphorylation during a physiological cell cycle, the extent/timing of mitotic substrate phosphorylation was quantified during the G2/M transition. This was done by taking the integral (Area Under Curve (AUC)) of phosphorylation between 50 and 100 minutes after release from G2 arrest. The second mitotic division (50-100 minutes) was used, as the dynamics of CDK activity accumulation are likely to be more physiological than immediately after release from a G2 arrest. Sites that are phosphorylated early in the G2/M transition will be phosphorylated for a longer proportion of the time course and as a consequence will also have a higher peak in quantified relative phosphorylation.

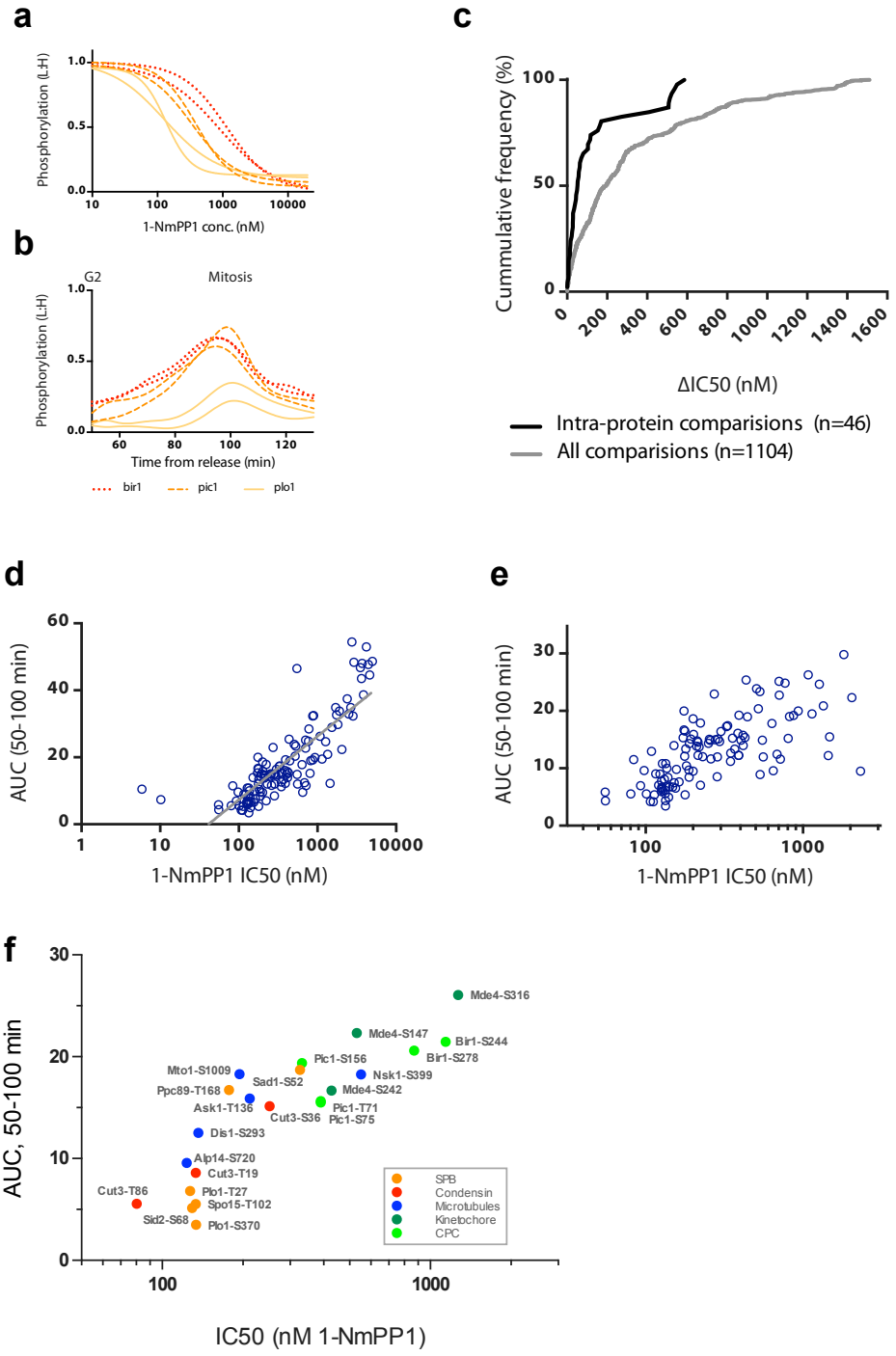

Figure 3.03 | Differences between mitotic phosphorylation sensitivity are smaller between sites in the same protein and correlate with their timing of phosphorylation at G2/M
a Examples of individual CDK substrate sites' relative phosphorylation (L:H) over a range of 1-NmPP1 concentrations (only sigmoidal fit plotted for presentation).
b Examples of individual CDK substrate sites' relative phosphorylation (L:H) during G2/M: 50-100 min after release from a G2 arrest (only Spline (smoothed line) plotted for presentation) (missing values imputed).
c Cumulative frequency plot of the ΔIC50 between all sites in multi-phosphorylated mitotic substrates (all comparisons) and between sites in the same mitotic substrate (intra-protein comparisons).
d-e Plot of the CDK substrate site sensitivity to CDK activity (i.e. 1-NmPP1 IC50) against the extent/timing of phosphorylation during G2/M (i.e. AUC (50-100 min), calculated from imputed and smoothed data) for **d** all quantified sites (i.e. R squared > 0.9), **e** all mitotic sites (two data points outside the axis).
f Plot of the example mitotic CDK substrate site sensitivity to CDK activity (i.e. 1-NmPP1 IC50) against the (i.e. AUC (50-100 min), calculated from imputed data).

This is because a larger proportion of cells, in an imperfectly synchronised culture (<50% synchrony), will be in a stage of the G2/M transition where such a site is phosphorylated. The AUC of sites correlates with the sensitivity of a site to CDK activity (1-NmPP1 IC50), therefore sites that are less sensitive to CDK activity are phosphorylated later during G2/M. This relationship holds true within mitotic substrates (Figure3.03e). Examples of mitotic substrates involved in different mitotic processes/functions are labelled in Figure 3.03f. These data are consistent with a model in which, as CDK activity rises during the G2/M transition, substrates are phosphorylated in an ordered manner according to their sensitivity to CDK activity.

In some multi-phosphorylated proteins there are significant differences between the IC50s of different sites, in contrast to the global tendency for site in the same protein to behave similarly. For example Cut3 has numerous CDK substrate sites in its N-terminus, of which Cut3-T19 phosphorylation has previously been shown to be essential for Cut3 nuclear import during mitosis (Sutani et al., 1999). Comparison of three of these sites (T19, S36 & T86) shows that they span a range of sensitivities to CDK activity, which correlates with the timing/extent of phosphorylation (AUC) during the G2/M transition (Figure 3.04a-c). The fact that these differences in sensitivity are recapitulated in a physiological cell cycle transition suggests they may be of biological significance in determining the timing of condensin related mitotic events.

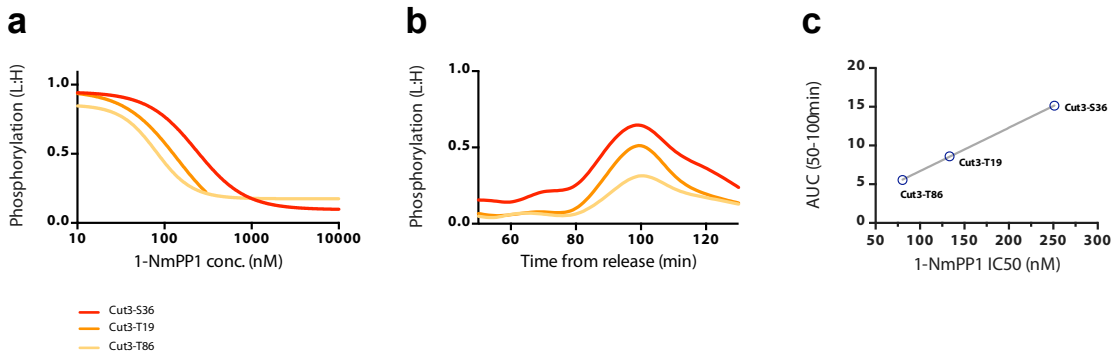

Figure 3.04 | Stepwise phosphorylation in the N-terminus of Cut3
a Cut3 phosphosites' relative phosphorylation (L:H) over a range of 1-NmPP1 concentrations (only sigmoidal fit plotted for presentation) (as in Figure 3.02).
b Cut3 phosphosites' relative phosphorylation (L:H) during the G2/M transition: 50-100 min after release from a G2 arrest (only Spline (smoothed line) plotted for presentation) (as in Figure 2.09)).
c Plot of Cut3 phosphosites' sensitivity to CDK activity (i.e. 1-NmPP1 IC50) against the extent/timing of phosphorylation during G2/M (i.e. AUC (50-100 min), calculated from imputed data).

3.2 Testing the causal relationship between CDK substrate phosphorylation and the organisation of cell cycle events

The observation that S-phase substrates are phosphorylated at lower CDK activities than mitotic substrates strongly suggests that the quantitative model for cell cycle progression works though differential sensitivity of substrate phosphorylation to CDK activity, but does not formally demonstrate it. Manipulating CDK activity to alter CDK substrate phosphorylation should allow the reorganisation of the cell cycle, if it is true that cell cycle events are ordered by differential substrate phosphorylation. There are two main predictions of a substrate phosphorylation threshold quantitative model for cell cycle progression:

(i) Premature high CDK activity in G1 cells should result in mitotic and S-phase substrate phosphorylation and initiate S-phase and mitosis simultaneously.

(ii) Removal of CDK activity should result in the dephosphorylation of S-phase substrates in G2 and re-phosphorylation of exclusively S-phase substrates at low CDK activity should initiate a second single round of DNA replication in the absence of mitosis.

It has been shown that modulating CDK activity can reorganise cell cycle events (Coudreuse and Nurse, 2010). To test how CDK substrate phosphorylation behaves in these reordered cycles, phosphorylation was quantified during six different cell cycle paths (reordered cycle A-C, released into (i) high or (ii) low CDK activity). A schematic of the experimental design for each of these reordered cycles is shown in Figure 3.05a. Reordered cycle A starts in a G2 arrest, cells are then treated with to 10 μM 1-NmPP1 (90 min) to remove CDK activity, after which cells are then release into high CDK activity (DMSO) or low CDK activity (1 μM 1-NmPP1). Reordered cycle B is the same as A, except cells are not treated with the 10 μM 1-NmPP1 and instead are maintained in a 1 μM 1-NmPP1 arrest. Finally reordered cycle C involves arresting cells in G1 for 150 minutes, by adding 10 μM 1-NmPP1 at anaphase after which cells are release into high CDK activity (DMSO)

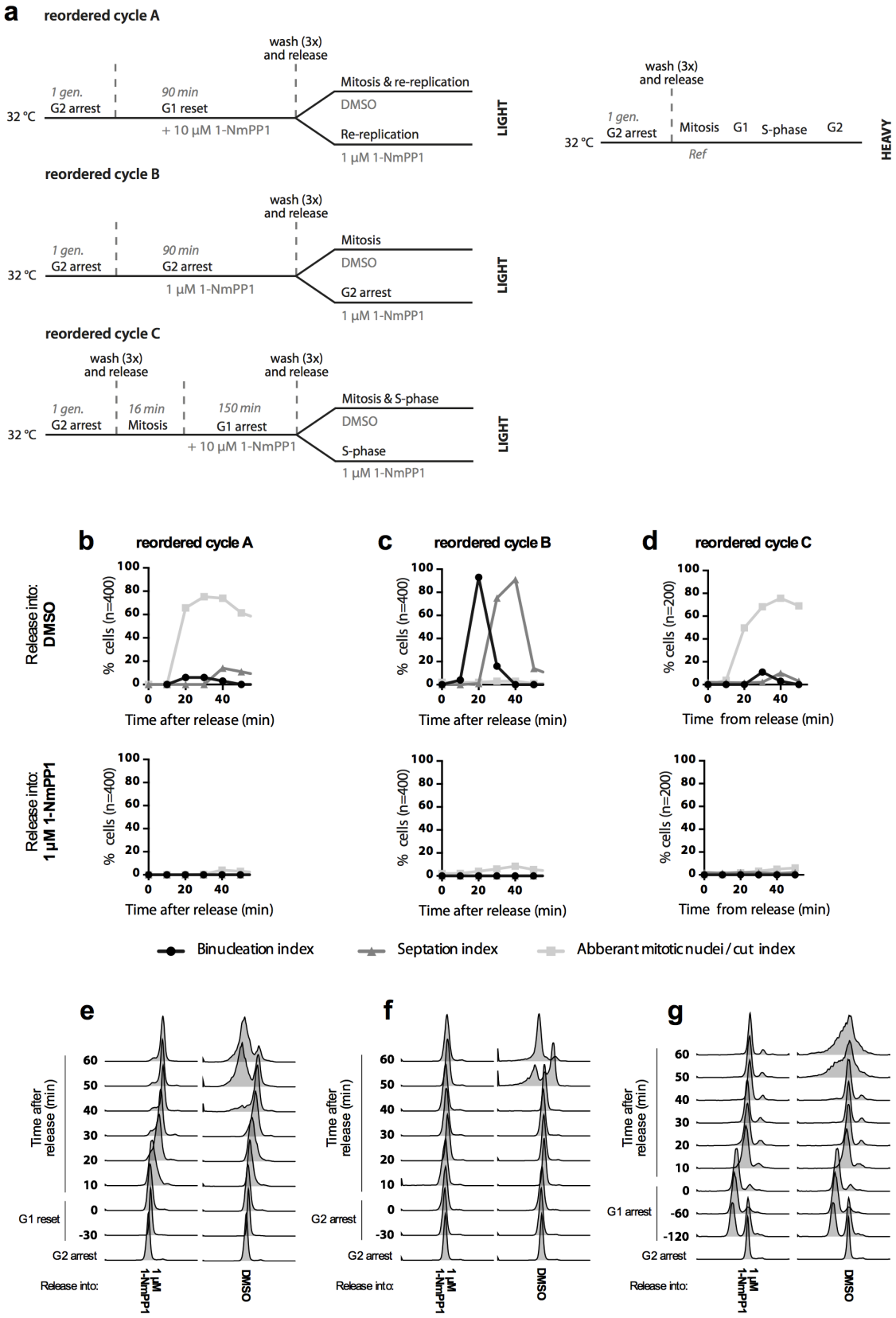

Figure 3.05 | Reordering of cell cycle events

Figure 3.05 | Reordering of cell cycle events (continued)
a Schematic of experimental design: unlabelled cultures (L) were reset in G1 (reordered cycle A (MS230)), arrested in G2 (reordered cycle B (MS230)) or arrested in G1 (reordered cycle C (MS108)). Cultures were then washed and released into DMSO or 1 μM 1-NmPP1 to direct cell cycle fate via six routes. Protein samples were recovered during the arrests/reset and 10 and 15 min after release. Protein samples were mixed with a common heavy labelled (H) reference (MS230, synchronised in mitosis). MS108 was used in reordered cycle C because of low synchrony in the release from G1 arrest in MS230.
Cell cycle fates are monitored in **b-g**.
b-d Chromosome and cell division quantified from DAPI and calcofluor stained cells during reordered cycle A-C (images of cells in Figure 3.06).
e-g DNA content, determined by FACS, during reordered cycle A-C.

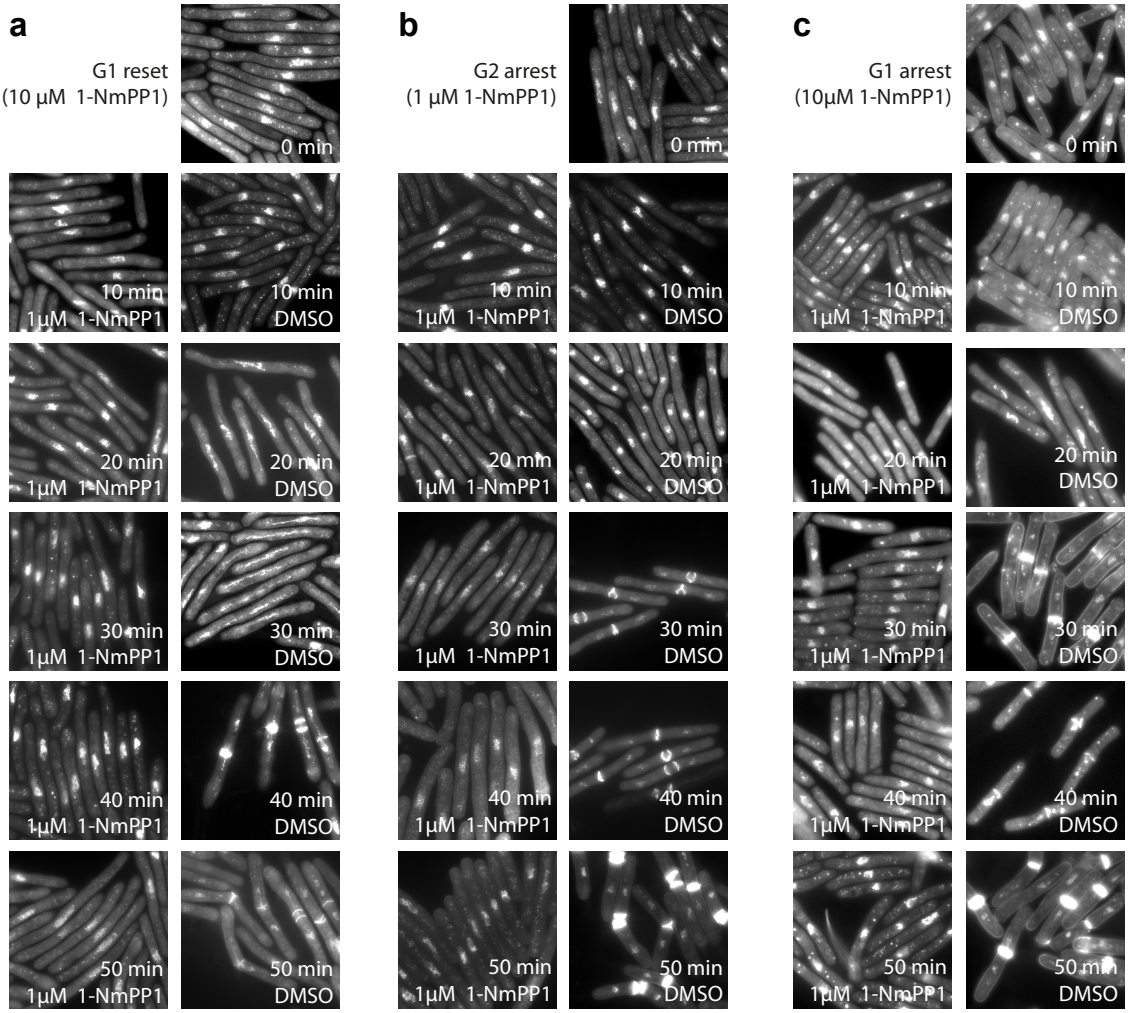

Figure 3.06 | Nuclear and cell division during reordered cell cycles
a-c DAPI (DNA) and calcofluor (cell septum) stained cells after wash & release (into 1 μM 1-NmPP1 or DMSO) for reordered cell cycle A-C. Time and wash & release conditions are labelled on each image.

or low CDK activity (1 μM 1-NmPP1). DNA content, chromosome division and cell division were monitored throughout (Figure 3.05b-g) (representative images of DAPI and calcofluor stained cells are shown in Figure 3.06). Protein extracts were taken during the reordered cycles (before and after release) and compared to a reference sample from cells synchronised in mitosis (Figure 3.05a).

3.2.1 Global CDK-mediated phosphorylation during reordered cell cycles

Figure 3.07 shows the median relative phosphorylation of S-phase and mitotic CDK substrates during these reordered cycles. In cells arrested in G1 (10 μM 1-NmPP1) S-phase and mitotic substrates are dephosphorylated (Figure 3.07c). Upon release into low CDK activity (1 μM 1-NmPP1) only S-phase substrates become fully phosphorylated and this initiates a single round of replication (Figure 3.05g, left panel (reordered cycle C)). When CDK activity is removed from G2 cells (10 μM of 1-NmPP1), S-phase substrates become dephosphorylated (G1-reset) and if low levels of CDK activity are restored (i.e. released into 1 μM 1-NmPP1) S-phase substrates become re-phosphorylated but mitotic substrates do not (Figure 3.07a, (reordered cycle A)). As S-phase substrates are re-phosphorylated, a single round of DNA replication is initiated and the G2 DNA content is re-replicated (Figure 3.05e). In contrast, when S-phase substrate phosphorylation is maintained at a high level throughout the experiment (Figure 3.07b) cells do not re-replicate (Figure3.05e, left panel (reordered cycle B)). This demonstrates that S-phase substrate phosphorylation controls the initiation of S-phase as long as cells have experienced a preceding G1-like state (G1-arrest or G1-rest) of low CDK activity to dephosphorylate S-phase substrates. This is consistent with an extensive body of work concerning the mechanisms of origin firing and licensing as discussed in the Introduction. If cells in a G1-like state (G1-arrest or G1-reset) are release into high CDK activity (DMSO), instead of low CDK activity (1 μM 1-NmPP1), S-phase substrates are still re-phosphorylated (Figure 3.07d&f (reordered cycle A&C)) and cells still undergo a round of DNA replication (Figure 3.05e&g right hand panel). This demonstrates that it is not only at a specific level of CDK activity at which S-phase is initiated, it is simply the net phosphorylation of S-phase substrates, at or above a specific CDK activity threshold.

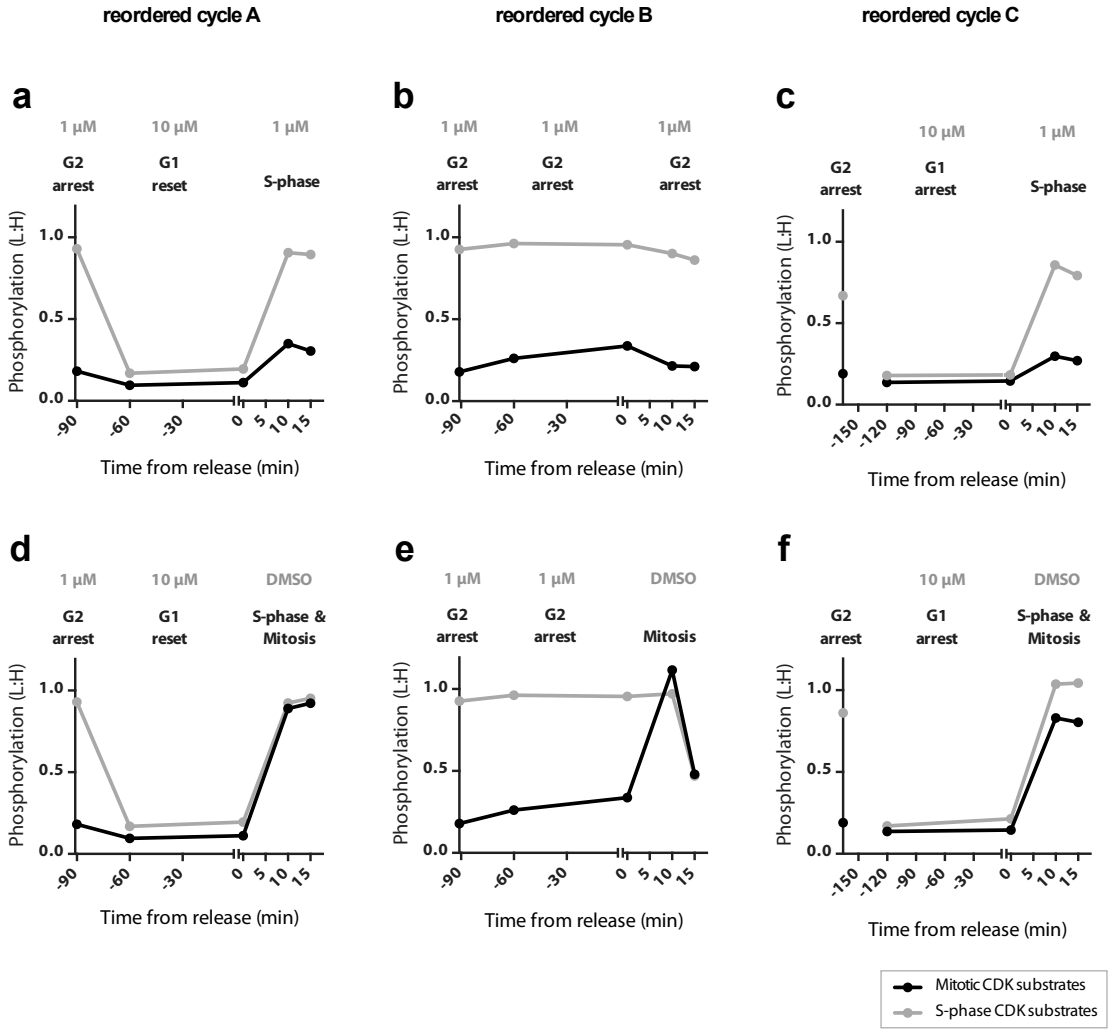

Figure 3.07 | Modulating CDK activity can direct differential mitotic/S-phase phosphorylation to reorder the cell cycle I
a-f Median phosphorylation (L:H) of mitotic and S-phase substrates during reordered cell cycle A-C. Corresponding cell cycle state (as defined in Figure 3.06) and 1-NmPP1 concentrations are annotated above the graph.

Cells re-set in a G1-like state released into high CDK activity (DMSO) also results in high mitotic substrate phosphorylation (Figure 3.07d), which drives cells into a mitotic-like state. Because cells are also undergoing a round of re-replication the overlap in S-phase and mitosis causes the majority of cells to present with aberrant mitotic phenotypes including mis-segregated chromosomes, stretched unsegregated chromosomes and mitotic catastrophe (i.e. cut) (Figure 3.05b) (Coudreuse and Nurse, 2010). If G2 cells that are not reset in G1 with 10 µM 1-NmPP1, and as such cannot re-replicate, are released into high CDK activity (DMSO) mitotic substrates become phosphorylated (Figure 3.07f) and normal chromosome segregation is initiated (Figure 3.05c upper panel). In contrast, when

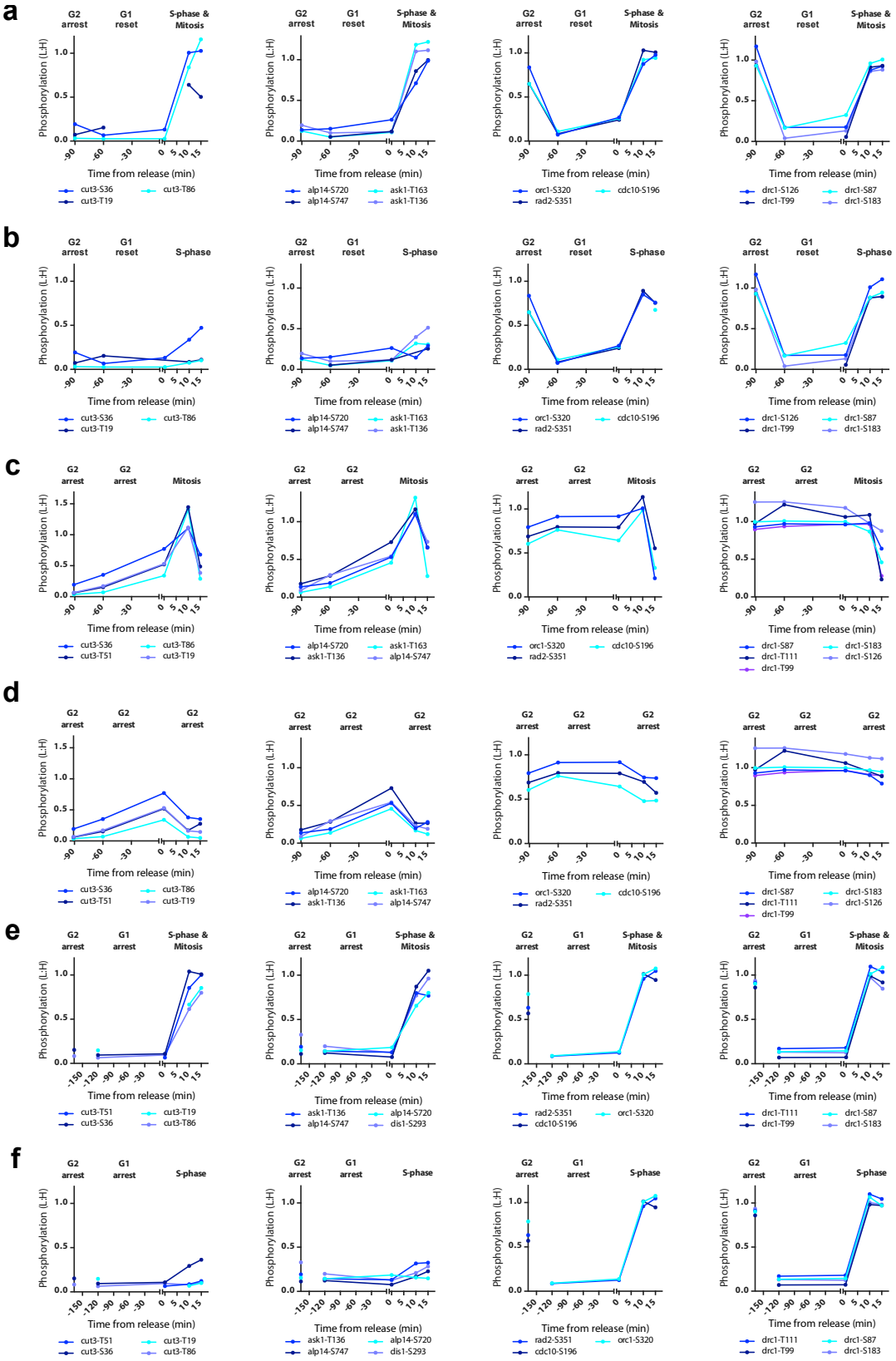

Figure 3.08 | Modulating CDK activity can direct differential mitotic/S-phase phosphorylation to reorder the cell cycle II

a-f Examples of individual CDK substrate sites' relative phosphorylation (L:H) during reordered cell cycles A-C. Corresponding cell cycle fates are annotated above the graph. Two left hand columns are examples of protein with mitotic functions. Two right hand columns are examples of proteins with S-phase functions.

mitotic substrates are not fully phosphorylated (Figure 3.07a&b), due to low CDK activity, cells do not enter mitosis (Figure 3.05b&c lower panel). Cells arrested in G1 (10 μM 1-NmPP1) that are released into high CDK activity (DMSO) also simultaneously fully phosphorylate S-phase and mitotic substrates (Figure 3.07f) resulting in the concurrence of S-phase and mitosis (figure 3.05d upper panel). The behaviours of S-phase and mitotic substrates are recapitulated at the level of individual substrates as can be seen by example of mitotic (Cut3, Ask1, Alp14 & Dis1) and S-phase related substrates (Orc1, Cdc10, Rad2 & Drc1) during these six conditions (figure 3.08).

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3.2.2 Candidate substrate phosphorylation during reordered cell cycles

These six re-ordered cycles were repeated using a strain harbouring tagged candidate substrates (Orc2 and Sid3) and candidate CDK substrate (Sld3, Orc2, Dis2 and Bir1) phosphorylation was assayed by Western blotting. The experimental outline is as in figure 3.05a. Chromosome division, cell division and DNA content are shown in figure 3.09, whilst figure 3.10 shows Orc2, Sld3, Bir1 and Dis2 phosphorylation as well as Cdc13-L-Cdc2 levels. When G2 cells are reset in G1 (10 μM 1-NmPP1) Orc2 and Sld3 are dephosphorylated and then re-phosphorylated as cells are released into 1 μM 1-NmPP1 (Figure 3.10a, left panel) resulting in cells undergoing DNA replication. When Sld3 and Orc2 phosphorylation is maintained high throughout the experiment (i.e. no G1-reset) re-replication does not occur. Bir1 and Dis2 are not maximally phosphorylated and cells do not enter mitosis in these conditions and Cdc13-L-Cdc2 continuously accumulates. When G1-arrested cells are released into 1 μM 1-NmPP1 only Sld3 and Orc2 are maximally phosphorylated and cells initiate S-phase (Figure 3.10c). When G1-arrested or G1-reset cells are released into high CDK activity (DMSO), Bir1, Dis2 Orc2 and Sld3 all become highly phosphorylated and S-phase and mitosis occur simultaneously (Figure 3.10c&b). After Bir1 and Dis2 phosphorylation Cdc13-L-Cdc2 levels drop precipitously indicating that the APC/C is activated. As Cdc13-L-Cdc2 is degraded all CDK substrates are dephosphorylated (Figure 3.10b, right panel) as in a normal mitotic exit (Figure 3.10a, right panel).

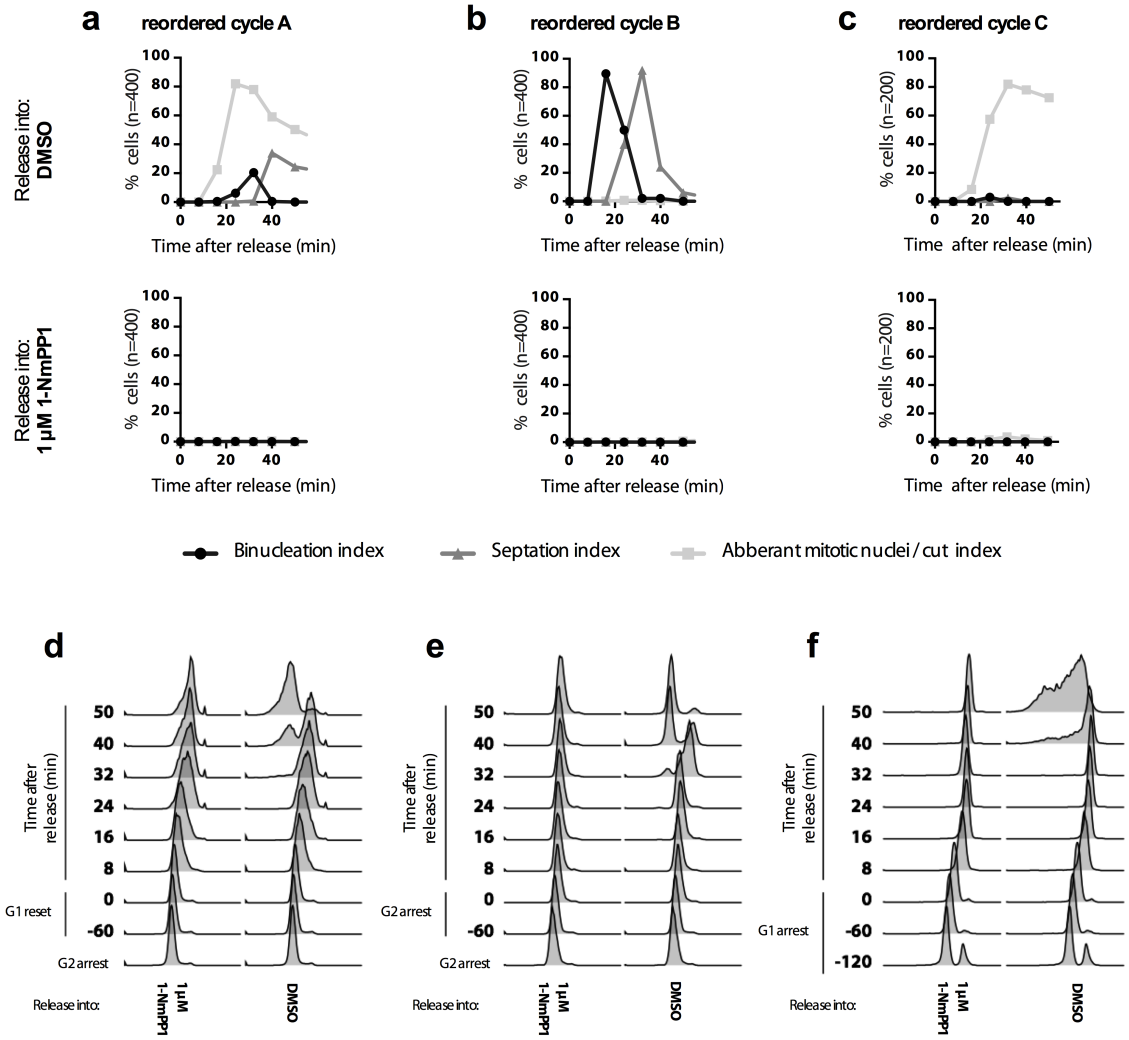

Figure 3.09 | Reordering of cell cycles events with epitope tagged substrates
Experimental design as in Figure 3.05a. Reordered cycle A&B were performed in YE4S (MS212) due to *orc2-3pk* dependent retardation of re-replication in EMM4S. Reordered cycle C was performed in EMM4S (MS132).
a-c DAPI and calcofluor stained cells quantification after release for reordered cycle A-C (images not shown).
d-f DNA content determined by FACS during reordered cycle A-C.

Interestingly Cdc13-L-Cdc2 levels are still able to start to rise after degradation resulting in rephosphorylation of Sld3 and Orc2 after the aberrant mitosis (Figure 3.10a, right panel). The phosphoproteomics describes the behaviour of site resolved phosphorylation on hundreds of CDK substrates (but often only one site per protein). Phosphorylation dependent band shifts have the benefit of assaying the net phosphorylation of an entire protein and demonstrates that Orc2, Sld3 and Bir1 exhibit stoichiometric changes in phosphorylation during these re-ordered cycles that are consistent with the observations from the phosphoproteomics data.

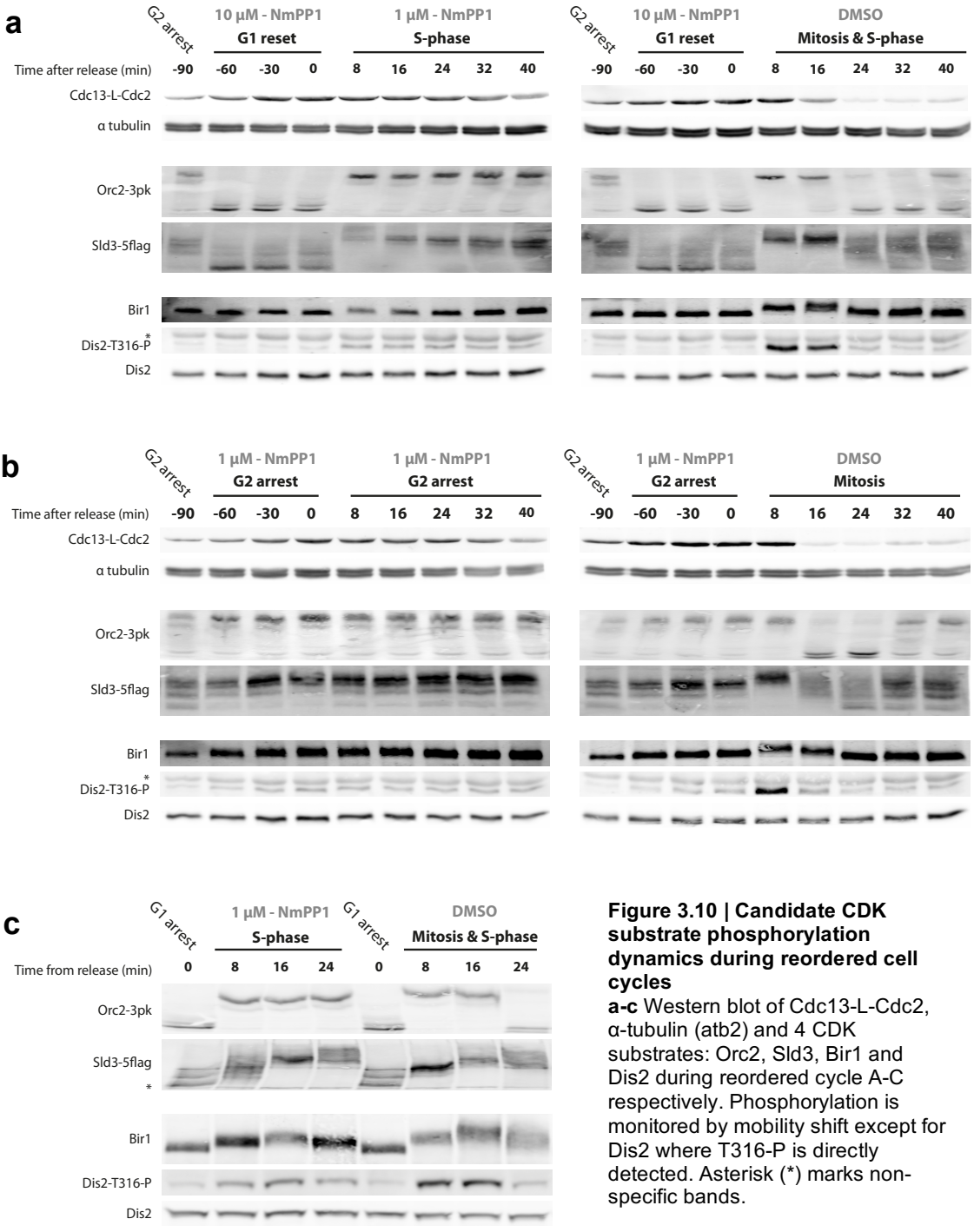

Figure 3.10 | Candidate CDK substrate phosphorylation dynamics during reordered cell cycles
a-c Western blot of Cdc13-L-Cdc2, α-tubulin (atb2) and 4 CDK substrates: Orc2, Sld3, Bir1 and Dis2 during reordered cycle A-C respectively. Phosphorylation is monitored by mobility shift except for Dis2 where T316-P is directly detected. Asterisk (*) marks non-specific bands.

Together these data demonstrate that CDK can be manipulated to alter the profile of differential CDK substrate phosphorylation. In these reordered cell cycles the differential phosphorylation of CDK substrates determines cell cycle fate. This supports the conclusion that there is a causal relationship between CDK activity, substrate phosphorylation and cell cycle fate and that there is no inherent directionality to the ordering of CDK substrate phosphorylation.

Interestingly some mitotic substrates become partially phosphorylated upon release into low CDK activity despite mitosis not being initiated. For example, a small increase in Dis2-T316 phosphorylation, and Bir1 mobility retardation, can be detected during the release from a G1-reset or G1-arrest into 1 μM 1-NmPP1. Looking at site resolved phosphorylation events in Figure 3.08, Cut3-T86 and Cut3-19 phosphorylation is maintained at a basal level in cells released into 1 μM 1-NmPP1, whereas Cut3-S36 phosphorylation (a site more sensitive to CDK activity) shows a small but significant increase. Furthermore, as cells grow during the G2 arrest and Cdc13-L-Cdc2 accumulates, mitotic phosphorylation sites rise significantly (including T19 in cut3 which is necessary for its nuclear import during mitosis) despite the fact that cells do not undergo nuclear division (Figure 3.8d). It is only when cells are released into DMSO and these sites phosphorylation hits maximal levels that mitosis is undertake. This may suggest that cells are robust in their ability to only undergo mitosis when the entire critical pool of CDK substrates is fully phosphorylated. This maybe because only specific less sensitive sites are critical for the full initiation of mitosis and a number of CDK phosphorylation events, normally phosphorylated during mitosis, are necessary but not sufficient to activate the machinery of mitotic division.

3.3 How flexible is the cell cycle machinery to alterations to the profile of CDK activity?

CDK inactivation has been show to be sufficient to relicense origin in *S. cerevisiae* (Noton and Diffley, 2000) consistent with the observation that transient inhibition of CDK permits re-replication (Coudreuse and Nurse, 2010). A transient CDK inhibition (10 μM 1-NmPP1) pulse of 90 minutes was used when Coudreuse and Nurse (2010) reported synchronous re-replication in G2 *S. pombe* cells. However, the dephosphorylation half lives of CDK substrates, including critical substrates for S-phase are almost all less than 10 minutes (Chapter 2). To investigate this further CDK activity was removed in G2 cells with 10 μM 1-NmPP1 pulses of varying length (30-90 min) and then released into 1 μM 1-NmPP1. The progression though the subsequent S-phase was determined by FACS analysis (Figure 3.11a). The final proportion of re-replicated cells is not dramatically different between the three pulse lengths (90 min: 81% >2C, 60 min: 80.7% >2C, 30 min: 71.4% >2C, 0 min 3.9% >2C) consistent with the conclusion that S-phase substrate dephosphorylation is sufficient to permit origin licencing. However shorter pulses result in slower replication perhaps indicating that factors downstream of CDK phosphorylation might be limiting. Numerous initiation factors besides CDK have been described in other systems as limiting the timing and/or progression though S-phase (Wu and Nurse, 2009, Mantiero et al., 2011, Collart et al., 2013).

Furthermore, when cells undergo a round of DNA replication after mitosis and a short G1, or after an extended G1-arrest, the majority of DNA synthesis is completed in a matter of 10-20 minutes, dramatically faster than after G2 cells are reset in G1 and released into 1 μM 1-NmPP1 (Figure 2.09d-f) (Coudreuse and Nurse, 2010). The differences in S-phase completion rate, may be due to one of two things: slower DNA synthesis rate (i.e. fork speed/processivity) or a lower number of origins being fired, probably due to reduced licensing efficiency. These differences may be due to a factor(s) that is regulated by progression through mitosis. In *Xenopus,* mitotic “conditioning” of the chromosomes is important for the subsequent cycle and involves increased replication factor recruitment to chromosomes and a shortening of the inter-origin distance during S-phase

(Lemaitre et al., 2005). Extending

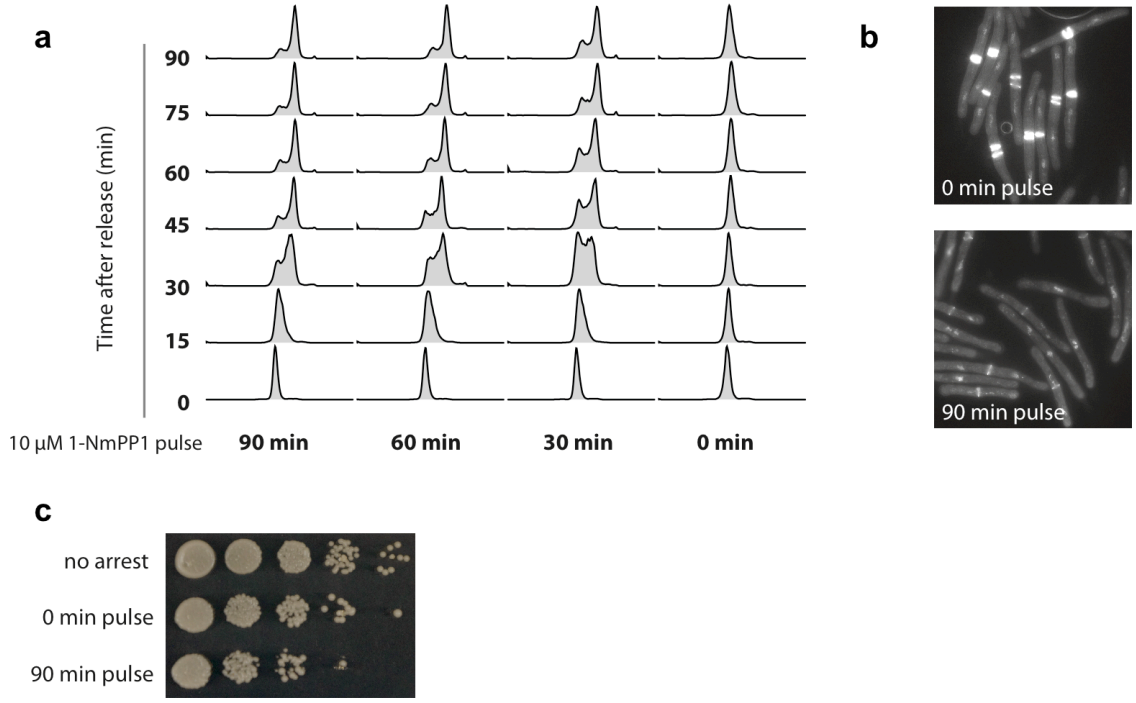

Figure 3.11 | Flexibility/robustness of the cell cycle to reorganisation by CDK activity
a DNA content determined by FACS after release from 10 µM 1-NmPP1 G1-reset into 1 µM 1-NmPP1. Experimental design for 90 min pulse is as in Figure 3.05a (re-ordered cycle A) (MS108). 10 µM pulse was delayed to shorten it (but not the timescale of the whole experiment) to 60, 30 or 0 min. Quantification of >2C DNA content: 90 min pulse: 81%, 60 min pulse: 80.7%, 30 min pulse: 71.4%, no pulse: 3.9%. 90 min after release into 1-NmPP1 cells exposed to a 90min 10 µM pulse or no 1-NmPP1 pulse were washed and released into media lacking 1-NmPP1.
b Representative images of DAPI and calcofluor stained cells 30 min after release into media lacking 1-NmPP1. Top panel: cells previously exposed to a 10 µM pulse (i.e. re-replicated) or no 10 µM pulse (i.e. no re-replication).
c Serial dilution (on YE4S plates) of cells plated one hours after release into 1-NmPP1 free media. No arrest: cells in exponential growth.

mitosis in *S. pombe* allows more, and less efficient, origins to accumulate ORC and alters the pattern of origin usage in the subsequent S-phase, indicating that passage though mitosis play a role in origin licensing (Wu and Nurse, 2009). For example, Cdc13 proteolysis might serve as such a requirement, given that Cdc13 transiently dissociates from chromatin at anaphase, coincident with when the MCM complex associates (Wuarin et al., 2002).

During the program of re-replication reported by Coudreuse and Nurse (2010) it appears that a single round of bulk DNA synthesis is completed but it is not clear if all aspects of the program of S-phase are completed appropriately and the post-replication chromosomes are competent for subsequent mitotic division as a diploid. To test this; cells in 1 µM 1-NmPP1 that had either been subjected to a preceding

90 min 10 μM pulse or no (0 min) 10 μM pulse, that did and did not re-replicate respectively, were released into media lacking 1-NmPP1. Cells that had not re-replicated segregated their chromosomes normally with a modest septation defect (Figure 3.11b). In contrast, cells that had re-replicated showed defects in chromosome segregation, and have a significant reduction in cell viability when plated as a serial dilution one hour after release (Figure 3.11b&c). This suggests that this program of re-replication is not as permissive to a subsequent mitosis as a normal S-phase. It is unclear why this is, but it could be due to failure to properly complete aspects of the DNA replication program, such as replication termination.

These two examples illustrate that despite CDK substrate phosphorylation being the major critical determinate for cell cycle progression, other aspects of the cell cycle machinery are optimally tuned to align with the physiological normality of the alternation of S-phase and mitosis.

3.4 Testing possible determinants of CDK sensitivity

As discussed in Chapter 2 given there are so few S-phase compared to mitotic substrates it is reasonable to consider then the exception to the rule with regards to phosphorylation timing. As such a few hypotheses regarding what determines the increased sensitivity of S-phase substrates were tested.

3.4.1 Consensus sites and distal short linear motifs

Figure 3.12a shows amino acid distribution around all S-phase, biphasic or mitotic phosphorylation sites and illustrates that S-phase substrates do not conform to the full CDK consensus site (S/T-P-X-K/R), but that there is a significant enrichment of the +3 lysine/arginine in the biphasic substrates compared to mitotic substrates. To test if any other global differences in CDK sensitivity require the full CDK consensus motif, the cumulative frequencies of all S/T-P-X-K/R site and all other CDK substrate sites were plotted (Figure 3.12b). This shows that there is no significant difference between the full and minimal CDK consensus site and furthermore there are also no significant differences between Threonine and Serine as the phosphorylated residue (Figure 3.12c).

To search for short linear motifs that may play a role in increasing S-phase substrate association with CDK, the protein sequences of five S-phase substrates (Orc1, Orc2, Sld3, Drc1 & Fkh2) were analysed using SLiMFinder, a bioinfomatic tool designed to identify short linear motifs enriched in a query list of proteins (Davey et al., 2010). All significantly over-represented motifs were related either to the CDK consensus site or a four amino acid motif: N-G-I/L/F-D/E. This motif was mutated to A-G-G-A in ectopically expressed and 5pk tagged Drc1, Sld3 and Orc2 constructs. Partial motifs lacking a position 4 Glutamate or Aspartate were mutated to A-G-G. Phosphorylation of the wild type and mutant (A-G-G-A) proteins was assayed by mobility shift in extracts from asynchronous cultures. Asynchronous cultures were analysed as *S. pombe* spend ~80% of the cell cycle during exponential growth, in S-phase and G2, when S-phase substrates are phosphorylated but mitotic substrates are not. Figure 3.12d shows that Drc1 and Sld3 have CDK dependent mobility shifts in the wild type and A-G-G-A mutant

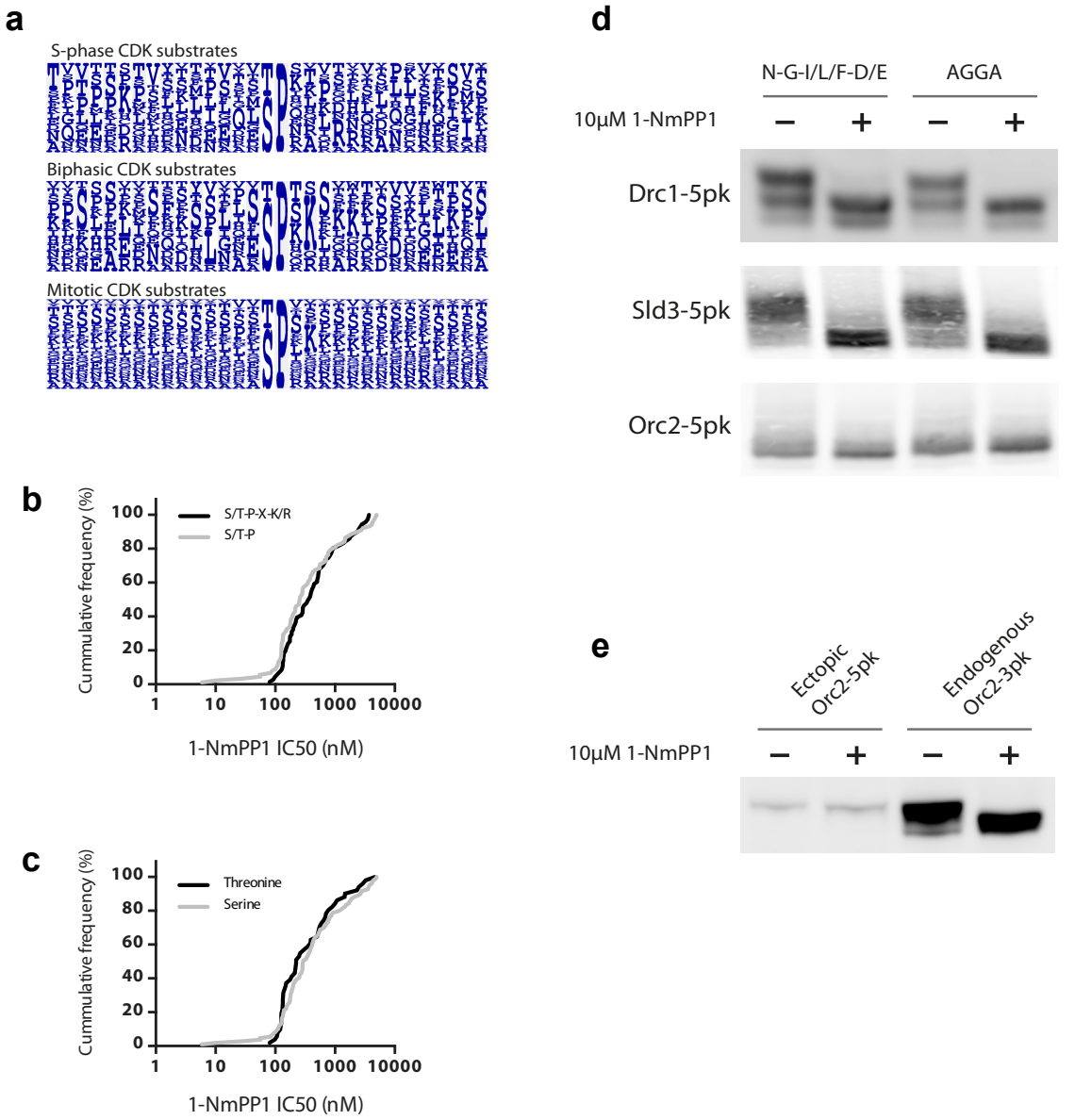

Figure 3.12 | Consensus sites and distal short linear motifs
a Consensus sites for S-phase, biphasic and mitotic substrates.
b&c cumulative frequency plot of CDK sensitivity (IC50 to 1-NmPP1) for the fully or minimal CDK consensus sites and Threonine or Serines phosphosites.
d Western blot of wild type (N-G-I/L/F-D/E) and mutant (A-G-G-A) Drc1-5pk, Sld3-5pk and Orc2-5pk with (10 μM 1-NmPP1, 20 min) and without (DMSO 1-NmPP1, 20 min) CDK inactivation in asynchronous exponential cultures. Ectopic constructs expressing wild type or mutant *nmt41-drc1-5pk*, *nmt41-orc2-5pk* and *nmt41-sld3-5pk* (de-repressed in minimal media – thiamine, >48 h, 32 °C) (all partial motifs (N-G-I/L/F) were also mutated to A-G-G).
e Phosphorylation dependent band shift of endogenously expressed Orc2-3pk and ectopically (nmt41) expressed Orc2-5pk with (10 μM 1-NmPP1, 20 min) and without (DMSO 1-NmPP1, 20 min) CDK inactivation in asynchronous exponential cultures + thiamine.

proteins, indicating that the N-G-I/L/F-D/E motif is not an essential determinate for Drc1 or Sld3 substrate phosphorylation. Surprisingly, ectopically expressed Orc2-5pk did not show any significant CDK dependent mobility shifts, even at sub-endogenous levels (Figure 3.12e).

3.4.2 Mitotic and S-phase substrates have similar dephosphorylation rates

It has been proposed that mitotic substrates might have faster dephosphorylation rates thus decreasing their net phosphorylation at interphase CDK activities (Coudreuse and Nurse, 2010, Uhlmann et al., 2011, Fisher et al., 2012). A prediction of this model is that mitotic substrates should be dephosphorylated with shorter half lives upon CDK inactivation. Figure 3.13a shows a cumulative frequency distribution of the phosphorylation half lives for S-phase and mitotic substrates after CDK inactivation in mitosis. There is no significant difference between the dephosphorylation rates of S-phase and mitotic substrates in these

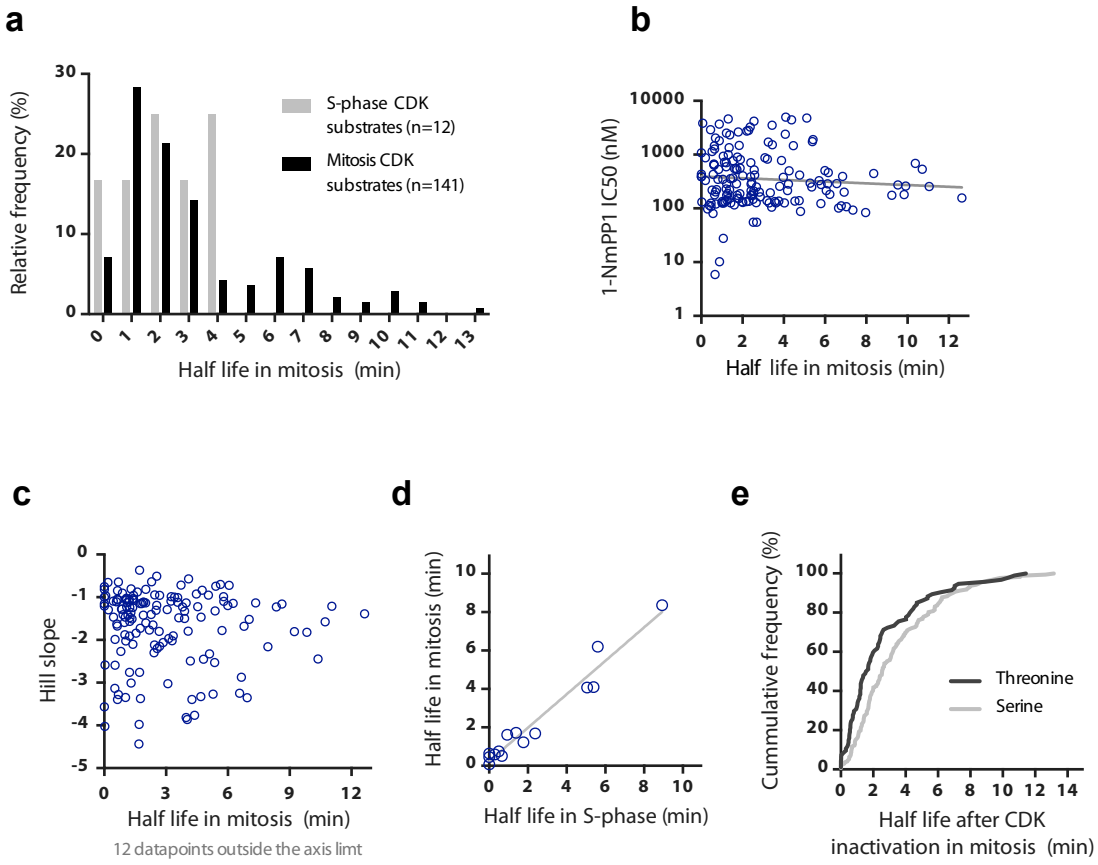

Figure 3.13 | CDK substrate dephosphorylation rates
a Histogram of phosphosite half life in mitosis after CDK inactivation for S-phase and mitotic substrates.
b Plot of CDK substrate phosphosite half lives after CDK inactivation in mitosis, against IC50 to 1-NmPP1. Sites with a one phase decay fit R squared > 0.95 and a sigmoidal fit R squared > 0.95 are plotted. Linear regression line: Y = -30.10*X + 856.9 (R squared = 0.005).
c Plot of CDK substrate site half lives, after CDK inactivation in mitosis, against hill slope from sigmoidal fit. (12 hill slope values lie below -5 and are not plotted)
d Plot of the phosphorylation half life during S-phase against half life calculated in mitosis. Sites with a one-phase decay fit R squared > 0.95 i after CDK inactivation n both S-phase and mitosis are plotted. Linear regression line: Y = 0.8710*X + 0.2361 (R squared = 0.954).
d Plot of the phosphorylation half life after CDK inactivation in mitosis and hill slope to CDK activity (i.e. IC50 to 1-NmPP1). Sites with a one phase decay fit R squared > 0.95 and a sigmoidal fit R squared > 0.95 are plotted. Linear regression line: Y = -30.10*X + 856.9 (R squared = 0.005).

experimental conditions. Consistent with this, phosphorylation half life does not correlate with the 1-NmPP1 IC50, or the hill slope for a given site (Figure 3.13b&c). These data indicate that differences between substrates in their sensitivity to CDK activity, or the abruptness of a sites response to CDK, cannot be explained exclusively by differential dephosphorylation rates of substrates by CDK-counteracting phosphatase(s) activity. S-phase substrate dephosphorylation rates are also unchanged between S-phase and mitosis, suggesting they are dephosphorylated by the same phosphatase throughout the cell cycle (Figure 3.13d). There is also a small general tendency for phospho-threonine residues to be dephosphorylated faster than phospho-serines (Figure 3.13e).

3.4.3 ORC stability influences Orc1 and Orc2, but not Sld3, phosphorylation

Fission yeast Orc2 is one of nine proteins that were identified as binding Cdc2 by yeast-2-hybrid (Leatherwood et al., 1996). CDK also associates with origins of replication in a Cdc13 dependent manner and can be displaced from origins using an Orc1 temperature sensitive (*ts*) mutant (Wuarin et al., 2002). Localisation to origins could increase the local concentration of CDK, resulting in preferential phosphorylation of S-phase substrates at origins. To test this, the phosphorylation state of three tagged endogenous S-phase substrates (Orc1, Orc2 and Sld3) was assayed in two ORC *ts* backgrounds: *orc1-4* (*cdc30-4*) and *orc2-2* (Figure 3.14a). ORC *ts* strains show a major 1C DNA content peak after shifting to the restrictive temperature indicating that there is a significant disruption of the ORC function in DNA replication (Figure 3.14b). Figure 3.14c shows that CDK dependent phosphorylation of Orc1 is significantly reduced in an *orc2-2* backgrounds at the restrictive temperature and Orc2 phosphorylation is modestly reduced in an *orc1-1* background, however Sld3 phosphorylation is unaffected by temperature shift in either ORC *ts* allele. Y15 phosphorylation in the Cdc2 moiety was also detected, quantified and normalised to Cdc13-L-Cdc2 levels and showed a small but consistent increase in Y15 phosphorylation upon shift to the restrictive temperature (Figure 3.13c&d). This could be because of the temperature shift itself or the ORC *ts* alleles resulting in DNA damage dependent checkpoint activation, and may indirectly influence substrate phosphorylation. To control for this, strains with and

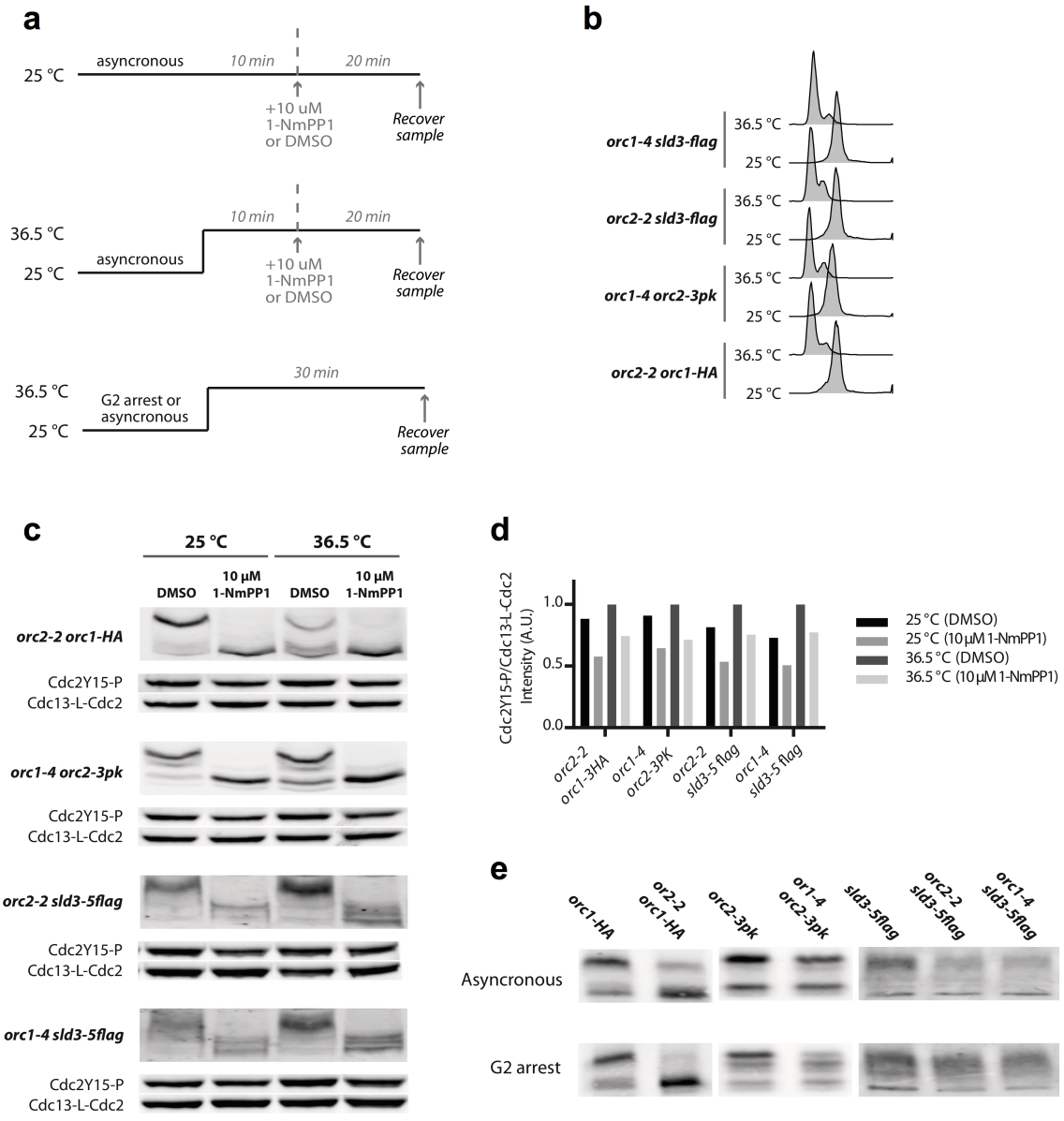

Figure 3.14 | Origin recognition complex stability is required for ORC protein phosphorylation but not Sld3 phosphorylation
a Schematic of experimental design: strains with temperature sensitive (*ts*) ORC alleles (*orc1-4* or *orc2-2*) and expressing tagged S-phase CDK substrates (*orc1-HA*, *orc2-3pk*, *sld3-5flag*) were grown at 25 °C. Asynchronous exponential cultures were either kept at the 25 °C (permissive temperature) (upper panel) or shifted to 36.5 °C (restrictive temperature) (middle panel). 10 min after temperature shift DMSO or 10 µM 1-NmPP1 was added and cell were recovered for protein samples 30 min after temperature shift.
b DNA content of cells 2 hours after shift to 25 °C or 36.5 °C.
c Western blots to detect CDK substrate phosphorylation by mobility shift of orc1-HA, orc2-3pk, sld3-5flag at 25 °C or 36.5 °C after DMSO or 10 µM 1-NmPP1 treatment. **c** also contains Western blots to detect Y15 phosphorylation of the cdc2 moiety and Cdc13-L-Cdc2 protein quantified in **d**
a Cells with and without ORC *ts* alleles in genes were either arrested in G2 or kept in asynchronous growth and shifted to 36.5 °C, protein samples were taken 30 min after shift (lower panel).
e Western blots to detect CDK substrate phosphorylation by mobility shift of Orc1-HA, Orc2-3pk, Sld3-5flag at 36.5 °C in asynchronous exponential cultures or cultures arrested in G2.

without the ORC *ts* background were shifted to the restrictive temperature from exponential growth or a G2 arrest (Figure 3.14, lower panel). Tagged strains without the ORC *ts* background showed normal phosphorylation at the restrictive temperature indicating that temperature shift *per se* does not affect phosphorylation. G2 arrested cells (where S-phase associated DNA damage will not occur) still showed a significant reduction in Orc1 phosphorylation in an *orc2-2* background, and a modest reduction in Orc2 phosphorylation in an *orc1-4* background, whilst Sld3 phosphorylation was unaffected (Figure 3.14e).

The fact that Sld3 phosphorylation is not influenced by the disruption of the ORC suggest that co-localisation of CDK and S-phase substrates at the ORC cannot be a universal mechanism to increase S-phase substrate sensitivity to CDK. The observation that the stability of different ORC components affects the phosphorylation of each other suggests that some of the determinates for ORC subunit phosphorylation exist at the level of the whole complex. The fact that an ectopic 5pk tagged version of Orc2 could not be phosphorylated by CDK but an endogenous 3pk tagged version could be because the larger 5pk tag disrupts incorporation of Orc2-5pk into the ORC complex, preventing it from being appropriately presented to CDK. Orc2-5pk phosphorylation is still not detected at sub-endogenous levels indicating that the lack of Orc2-5pk phosphorylation is not because it is ectopically expressed in excess of the endogenous ORC subunits (Figure 3.12e).

3.5 Discussion

The data presented in this chapter demonstrates that the timing of CDK substrate phosphorylation is determined by differential sensitivity to CDK activity with S-phase substrates more sensitive to CDK activity than mitotic substrates. The fact that the correlation between CDK activity, substrate phosphorylation and cell cycle fate is maintained outside of the physiological cell cycle, in re-ordered cell cycles, is consistent with two causal relationships. Firstly, low CDK activity results in S-phase substrate phosphorylation and thus bringing about DNA replication. Secondly, high CDK activity brings about mitotic substrate phosphorylation initiating mitosis. The ability of CDK activity to control substrate phosphorylation, and in turn, cell cycle events, is not contingent on the cell cycle history of the cell and argues that there is no inherent directionality to the order of substrate phosphorylation of the *S. pombe* cell cycle. S-phase substrates are also more ultrasensitive to CDK activity than mitotic substrates. S-phase substrates may have more abrupt response to CDK activity to ensure the transition from G1 to S-phase is switch like. This may not be required in mitotic substrates as the rise in CDK activity from G2 to M is thought to be significantly greater and more abrupt. So the switch like commitment to mitosis may be achieved via the non-linear dynamics of CDK activity, as opposed to the non-linear response of all substrates to CDK activity.

There is also a third category of CDK substrates that have an intermediate sensitivity to CDK activity and increase in phosphorylation significantly at both S-phase and mitosis. These biphasic substrates peak in G2 just before mitosis, indicating that they are sensitive to changes at a third threshold of CDK activity during G2. The exact significant of the timing of these biphasic substrates is not clear but may be involved in the completion of S-phase or the preparation of chromosomes for mitosis. Rad2 (Flap endonuclease, involved in Okazaki fragment maturation), Mis4 (the Cohesin loader) and Mcm10 (DNA initiation factor) all have phosphorylation events that show this biphasic behaviour, suggesting that the termination of DNA replication and/or remodelling of Cohesin or Cohesin loading machinery may be controlled by this G2 phosphorylation. Consistent with this, the human Flap endonuclease (Fen1) is phosphorylated by Cdk2 to inactivate it and disrupt its interaction with PCNA (Henneke et al., 2003). In *S. pombe* Rad2, the

PCNA interaction peptide (336-344) sits seven amino acids N-terminal to the detected CDK site (S351), suggesting it may also function to disrupt the Fen1-PCNA interaction. It might be the case that cells that are manipulated to re-replicate, as described above, simultaneously phosphorylate S-phase and biphasic substrates preventing the completion/termination of S-phase in some respects. This could explain why they are not all able to undergo a physiological mitosis as a diploid following re-replication.

3.5.1 Possible determinates of sensitivity to CDK activity

How these important differences in sensitivity are biochemically determined is unclear. The fact that sites with the same protein tend to have more similar CDK sensitivities to each other suggests that the determinants for sensitivity are more likely to be due to protein-wide properties, not site autonomous properties. This could require ancillary factors (such as Cks1) that promote processive phosphorylation reactions on multisite substrates. The inter-dependency of ORC subunit phosphorylation also indicates that multiple proteins within the same multi-protein complex can function together to determine complex-wide properties and that one subunit of a complex can directly contribute to the phosphorylation status of another. There are multiple mechanisms by which such differences in sensitivity could be achieved.

Differential specificity for residues surrounding the phosphorylation site or increased biochemical affinity due to a docking interaction with a substrate are the simplest determinants to envisage. A simple hypothesis would be that early substrates conform to full CDK consensus site (S/T-P-X-K/R) whilst mitotic substrates only adhere to the minimal site (S/T-P) (Koivomagi et al., 2011b). However, this is not the case, and in fact the full CDK consensus sequence is under represented in S-phase substrate sites. Biphasic substrate sites are strongly enriched in the +3 K/R, suggesting that the full consensus site may be necessary for their increased sensitivity compared to mitotic substrates, but cannot be considered sufficient given many mitotic substrates also conform to the S/T-P-X-K/R consensus.

Kinase-substrate docking interactions are often distal to the phosphorylation site and have been described for a wide range of regulatory kinases (e.g. ERK, MEK, JNKs, PHK, GSK3 and PDK1) (Ubersax and Ferrell, 2007). In its simplest form a docking reaction would increase the local concentration of a substrate around a kinase, reducing the Km of a substrate. Docking motifs could also position substrate sites with respect to the active site, to decreasing a substrates Kcat or function to allosterically stimulate kinase activity, as has been reported for MAPK and ERK (Chang et al., 2002) (Lee et al., 2004). The only candidate docking motif identified amongst S-phase substrates (N-G-I/L/F-D/E) was dispensable for substrate phosphorylation. An as of yet unidentified docking motif seems like the most likely determinant for differences in sensitivity to CDK activity. In an analogous manner binding to a common scaffold or platform could perform a similar function as a motif that directly docks a kinase to a substrate (Ubersax and Ferrell, 2007). The possible role of the ORC complex as a platform to increase the local concentration of CDK and S-phase substrates to enhance S-phase substrate phosphorylation was tested but was not important for the phosphorylation of a major S-phase substrate: Sld3.

As discussed in the Introduction Cyclin-CDK localisation changes dynamically during the cell cycle and the spatial restriction of a cyclin can limit is ability to phosphorylate sub-pools of substrates. During mitosis in *S. pombe* CDK is localised across the nucleus and enriched on the spindle and chromatin (Decottignies et al., 2001). CyclinB1-Cdk1 auto-phosphorylates Cyclin B1 at multiple N-terminal Serine residues, which is important for the association of Cyclin B1 with chromatin and the spindle (Santos et al., 2012). A hypothesis for how the timing of mitotic substrates could be achieved is as follows. Outside of mitosis Cyclin-CDK does not see mitotic substrates, then once CDK activity surpasses a specific threshold it auto-phosphorylates directing itself to the chromatin and spindle to co-localise with mitotic substrates resulting in their phosphorylation. However, there are numerous examples of mitotic substrates that are cytoplasmic or nucleolar and the majority of S-phase substrates also interact with chromatin. It is equally plausible to speculate that subcellular co-localisation of CDK and its substrates could be of little significance: if the exchange cycle of CDK or CDK substrates on/off specific subcellular structures is dynamic enough then the

transient association of CDK and a substrate when off those structures may be sufficient to permit net substrate phosphorylation, especially if the dephosphorylation rate is slow comparative to the phosphorylation rate or if phosphatase activity were also spatial regulated. This raises the question as to whether all the dynamic changes in the local concentration of Cyclin-CDK actually have any meaningful impact on the phosphorylation of substrates specifically at those subcellular locations. The fact that a CDK activity reporter in human cell lines does not seem to show an increased read-out at locations where CyclinB1 becomes enriched during early mitosis hints this may not be the case (Gavet and Pines, 2010a). The experimental tethering of CDK and CDK-counteracting phosphatases to certain subcellular locations should allow the contribution of the subcellular localisation of CDK substrates to be probed more systematically.

Which phosphatase(s) counteract CDK phosphorylation in fission yeast is not yet fully clear. Clp1 certainly plays a significant role, although it possible this is predominantly during mitotic exit. It has been reported that phospho-threonine is dephosphorylated significantly slower by *S. cerevisiae* Cdc14 (Clp1 ortholog) than phospho-serine (Bremmer et al., 2012). However, CDK substrate sites that are phospho-threonines are dephosphorylated slightly faster than phospho-serines after CDK inactivation. This suggests that other phosphatases besides Clp1 are also operating to antagonise CDK activity in *S. pombe*. It has been hypothesised that a difference in timing of S-phase and mitotic substrate phosphorylation could be due to specific phosphatase activities that antagonises CDK phosphorylation on mitotic but not S-phase substrates (Coudreuse and Nurse, 2010, Uhlmann et al., 2011, Fisher et al., 2012). However, the dephosphorylation rate of mitotic substrates is no faster than that of S-phase substrates, arguing against this hypothesis. An important caveat to this conclusion is that phosphatases are down regulated in mitosis (see Introduction (section 1.3)), which is when the dephosphorylation rates are measured. It is plausible that the dephosphorylation rate of mitotic substrates in interphase is dramatically higher and imparts a threshold for greater CDK sensitivity until cells commit to mitosis, when phosphatases are inactivated and dephosphorylation rates drop. However, phosphate turnover is measured after CDK inactivation, when CDK regulated phosphatases should become de-repressed. If mitotic substrates are completely

refractory to interphase phosphatase activities in these experimental conditions, then interphase phosphatase activity could be the exclusive determinant for the timing of mitosis substrates, as long as mitotic substrates were dephosphorylated with half lives significantly shorter than the least stable S-phase substrate (< 1 minute). Especially given that dephosphorylation rates of S-phase substrates does not change between S-phase and mitosis. The fact that S-phase sites are dephosphorylated with the same rate in mitosis and S-phase suggests that whichever phosphatase(s) is dephosphorylating S-phase substrates is not differentially regulated between S-phase and mitosis. Given the span of dephosphorylation rates quantified here it is also important to consider different substrates may be able to achieve the same net phosphorylation with different dephosphorylation and phosphorylation rates as long as the ratio between the two rates (or some parameters related to them) are kept at a constant ratio.

Substrate competition could also play a role in resolving the phosphorylation of different substrate apart from one another. Substrate competition has been shown to contribute *in vitro* to the dynamics of Wee1 inactivation by CDK phosphorylation at T150 (Kim and Ferrell, 2007). The phosphorylation of reconstituted Wee1 by CDK is more ultrasensitive when co-incubated with cell lysates or other recombinant CDK substrates. It also significantly increases the concentration of CDK required to reach half maximal Wee1 T105 phosphorylation (Kim and Ferrell, 2007). This demonstrates that more sensitive substrates with a lower Km could control both the timing and the non-linear dynamics of the phosphorylation of less sensitive CDK substrates via competition. Interestingly phosphorylated α-endosolphine, the inhibitor of PP2A-B55, has recently been shown to also be a substrate of PP2A-B55 and function via an “unfair competition” mechanism. Phosphorylated α-endosolphine has a very low Km and Kcat meaning it binds PP2A-B55 very well and is dephosphorylated slowly preventing the enzyme working on other substrates. The main consequence of this is that PP2A-B55 does not dephosphorylate its wide range of other substrates until α-endosulphine is dephosphorylated and displaced from the PP2A-B55 active site (Williams et al., 2014). A similar situation could be envisaged in which S-phase substrates compete for CDK to prevent phosphorylation of mitotic substrates at low CDK activity levels. The constant turn-over of S-phase substrate phosphorylation could allow a steady-

state in which S-phase substrates are continuously being phosphorylated and occupy the small number of active CDK molecules early in the cycle, although it seems unlikely that S-phase substrates will have Michaelis-Menten parameters as extreme as that of phosphorylated α-endosulphine.

Given that a molecular precedent exists for all of these mechanisms in regulating kinase-substrate specificity, it is plausible that different substrates use different mechanisms, possibly combinations of alternate mechanisms, to hone their specific sensitivity to CDK activity in the cell. Such a scenario would make it incredibly challenging to assess globally how CDK-substrate dynamics are determined in anything other than a case-by-case basis.

3.5.2 Staggered substrate phosphorylation during the G2/M transition

In addition to the ordering of CDK substrate phosphorylation across the cycle, CDK substrates are progressively phosphorylated during the G2/M transition. The extent/timing of phosphorylation during G2/M, of a mitotic substrate site, correlates with its sensitivity to CDK indicating that rising CDK activity may also order the progression through G2/M as well as between S-phase and mitosis. What determines these smaller differences in sensitivity may come down to subtle structural and sequence differences that impact the affinity and reaction kinetics of CDK and CDK-counteracting phosphatases towards specific substrates. There is no significant difference in the sensitivity to CDK between the full and minimal consensus site or between Serine or Threonine indicating that these phosphorylation site proximal factors cannot be generally responsible for these small differences.

The ordered phosphorylation of CDK substrates during the G2/M transition has been previously reported in somatic extracts, where Wee1 is phosphorylated at low Cyclin B1 concentrations, followed by Securin, Cdc25, and finally Cdc27, the APC/C subunit. The ordered phosphorylation of Wee1 and Ccd25 is thought to contribute to the non-linear dynamics of CDK activation (Deibler and Kirschner, 2010). This graded phosphorylation of CDK substrate is far less pronounced in

Xenopus egg extracts (Georgi et al., 2002). Furthermore, CDK activity, measured in single cells by the net phosphorylation of a CDK activity biosensor rises progressively as cells transverse the G2/M transition until after NEBD. As the biosensor phosphorylation increases cells undergo sequential mitotic events (Gavet and Pines, 2010b). In cells unable to attain the highest mitotic levels of biosensor phosphorylation, later mitotic events (e.g. NEBD, chromosome alignment and cytokinesis) fail to occur, while earlier events (e.g. cell rounding and Cyclin B1 nuclear import) do occur (Gavet and Pines, 2010b). These data are consistent with a model where CDK substrates are phosphorylated in an orderly manner during G2/M at specific CDK activity thresholds, due to differential sensitivity to CDK activity, and that this ordering of substrate phosphorylation is important in ordering the events of mitosis. Alternatively, early G2/M phosphorylation could be necessary but not sufficient for the associated mitotic event if there were no physiological consequence to it being phosphorylated slightly earlier than required, whereas phosphorylation of other substrates may have to be precisely timed to ensure the timely progression through mitotic events. In the former scenario, there may be no selection for a precise sensitivity to CDK activity, only a pressure to evolve phosphorylation that occurs at or before the corresponding mitotic event.

Figure 3.03f shows examples of mitotic substrates that span the range of sensitivities to CDK activity. For example, phosphorylation that function to inhibit proteins before anaphase onset (e.g. Nsk1) are phosphorylated earlier than protein involved in mitotic spindle assembly (e.g. Alp14 and Dis1) or chromosome condensation (e.g. Cut3). These timing differences could function to ensure that factors involved in anaphase are fully inhibited first so that they cannot disrupt the events of early mitosis. SPB proteins (Sad1, & Ppc89) are also phosphorylated earlier and at lower CDK activities, which may implicate them in the very early mitotic event of nuclear SPB insertion (Ding et al., 1997, McCully and Robinow, 1971). Consistent with this Kms2 phosphorylation has an AUC of 16, similar to that of Sad1 and Ppc89, although no IC50 could be adequately calculated. Kms2 is an essential SPB-localised protein and its depletion results in aberrant SPB nuclear insertion (Walde and King, 2014). In contrast, other SPB proteins (Sid2 and Spo15) involved in the later events of cytokinesis are phosphorylated significantly later.

One, initially counter-intuitive, observation is that phosphorylation of Plo1 by CDK occurs later than most of the above proteins, despite the fact that Plo1 localisation to the SPB is thought to be one of the earliest mitotic events (Mulvihill et al., 1999). Plo1 recruitment to the SPB is dependent on its polo-box domain (PBD) (Reynolds and Ohkura, 2003) likely via the binding to SPB phospho-epitopes. As such the early phosphorylation of SPB proteins, as opposed to Plo1 itself, is probably what targets Plo1 to the SPB. Consistent with this, phosphorylation of Cut12 has been heavily implicated in Plo1 recruitment to the SPB (Grallert et al., 2013a) and Kms2 depletion has been shown to slow the rate of Cut12 and Plo1 accumulation at the SPB (Walde and King, 2014). The later phosphorylation of Plo1 may specifically function to further activate/regulate Plo1 for the later events of cytokinesis, in which Plo1 is also involved (Almonacid et al., 2011, Mulvihill and Hyams, 2002, Ohkura et al., 1995). The separation of the dual functions of Plo1 in chromosome segregation and septation was uncovered by the overexpression of mutations in the PBD that cause septation defects, but not the mitotic arrest observed when wild type Plo1 is overexpressed (Reynolds and Ohkura, 2003). Aurora kinase (part of the CPC) is involved in ensuring the proper bi-orientation of chromosome attachments at a kinetochore (Ruchaud et al., 2007) and the phosphorylation of Pic1 and Bir1 (CPC subunits) controls its localisation to the kinetochore (Pereira and Schiebel, 2003, Tsukahara et al., 2010)). Pic1 and Bir1 phosphorylation precedes the phosphorylation of substrates involved in spindle assembly and attachment, possibly to ensure that Ark1 is at the kinetochore before the spindle starts to be constructed, and the first spindle attachments forms, to allowing it to monitor and remodel all spindle attachments throughout mitosis. The exact significance of the timing of these phosphorylation events at present is speculative, but a surprising amount of sense can be made of them in light of what is known about their involvement in the various events of mitosis.

Early G2/M phosphorylation, which is more sensitive to CDK activity, could also function to inhibit phosphorylation of later events via substrate competition to buffer the timing of later mitotic events. Some CDK phosphorylation may not functionally regulate the target substrate and could simply function to compete with other substrates to buffer and delay their phosphorylation until later in the cycle. CDK phosphorylates a large number of substrates, and it is conceivable that some

proteins have “accidentally” or “passively” evolved CDK phosphorylation sites, in the absence of negative selective pressure. Recent work in *S. cerevisiae* has shown that a significant expansion of the Ime2 kinase’s specificity has no major dominant consequences indicating that cells are able to tolerate a significant amount of promiscuous phosphorylation (Howard et al., 2014). If there were a sufficient pool of such “passenger” CDK substrates, lacking any actual CDK regulated functions, they may have been maintained as substrates to regulate the phosphorylation of other substrates, through substrate competition.

Sites within the same protein are more likely to behave like each other but there are exceptions to this general tendency. For example, three sites in the N-terminus of Cut3 are phosphorylated sequentially at rising CDK activity thresholds. This strikes a notable resemblance to the description of CDK dependent phosphorylation of *Xenopus* Wee1. Wee1 phosphorylation occurs at five N-terminal sites; T104 and T150 are highly conserved and are required for Wee1 inactivation at mitotic entry (Kim et al., 2005). Three other sites, S38, T53 and S62, are poorly conserved, non-essential for Wee1 inactivation and are phosphorylated before T150 (Kim et al., 2005). Kim et al. (2007) demonstrated that these non-essential sites act as decoy targets for CDK, competing with and delaying T150 phosphorylation and also generate a more ultrasensitive output of T150 phosphorylation. An equivalent mechanism could be responsible for the ordered phosphorylation of sites in Cut3. The exact significant of multisite phosphorylation in Cut3 has not been investigated but could be significant in the exact dynamics of CDK regulated Cut3 nuclear influx during mitosis (Sutani et al., 1999). Unfortunately, Wee1 phosphorylation was not detected in this dataset so the potential conservation of this behaviour between *Xenopus* Wee1 and *S. pombe* Wee1 could not be assessed.

Chapter 4. The contribution of G1/S-phase cyclins

All the experiments described in Chapter 2 and 3 were preformed using strains with a genetic background lacking endogenous cyclins (*cdc13-L-cdc2 Δ2 Δ13 ΔCCP*). *S. pombe* has three cyclins besides Cdc13 that associate with CDK during the cell cycle and contribute to progression through G1 and S-phase: Puc1, Cig1 Cig2 (Booher and Beach, 1988, Bueno et al., 1991, Bueno and Russell, 1993, Connolly and Beach, 1994, Martin-Castellanos et al., 2000). As discussed during the Introduction, G1 and S-phase cyclins in *S. cerevisiae* have enhanced biochemical affinity towards a subset of CDK substrates. However, the global contribution of these associations to *in vivo* phosphorylation has not been extensively studied, and there is very little work on *S. pombe* cyclin specificity. In this chapter I present the quantification of CDK substrates during a S-phase arrest in the absence and presence of S-phase cyclins.

4.1 Testing the role of Cig1, Cig2 and Puc1

To test the differential contribution of the *S. pombe* cyclins to CDK substrate phosphorylation, the phosphoproteome was analysed in three backgrounds: wt (*cig1+ cig2+ puc1+*), Δ*CCP* (*cig1Δ cig2Δ puc1Δ*) and *cdc13-L-cdc2 Δ2 Δ13 ΔCCP*. In asynchronous exponential growth wild-type *S. pombe* shows no G1 DNA content peak (1C) because in nutrient rich conditions cytokinesis occurs after DNA replication. In contrast, Δ*CCP* shows a pronounced G1 peak whilst *cdc13-L-cdc2 Δ2 Δ13 ΔCCP* shows a small G1 population (Figure 4.01a). In all three strains Cdc2 has the F84G mutant so 1-NmPP1 could be used to synchronise cells in G2. Because endogenous Cdc2 does not fully tolerate the F84G mutant, an additional mutation was used that recues the associated defects (Aoi et al., 2014). This is not required in the Cdc13-L-Cdc2 construct, which does not present with F84G associated defects (Coudreuse and Nurse, 2010). Light labelled cultures (wt, Δ*CCP* and *cdc13-L-cdc2 Δ2 Δ13 ΔCCP*) were released from G2 into hydroxyurea (HU) in order to deplete nucleotide pools and arrest cells in S-phase (Figure 4.01b). In the absence of HU, wt cells undergo bulk DNA synthesis 30-40 min after release,

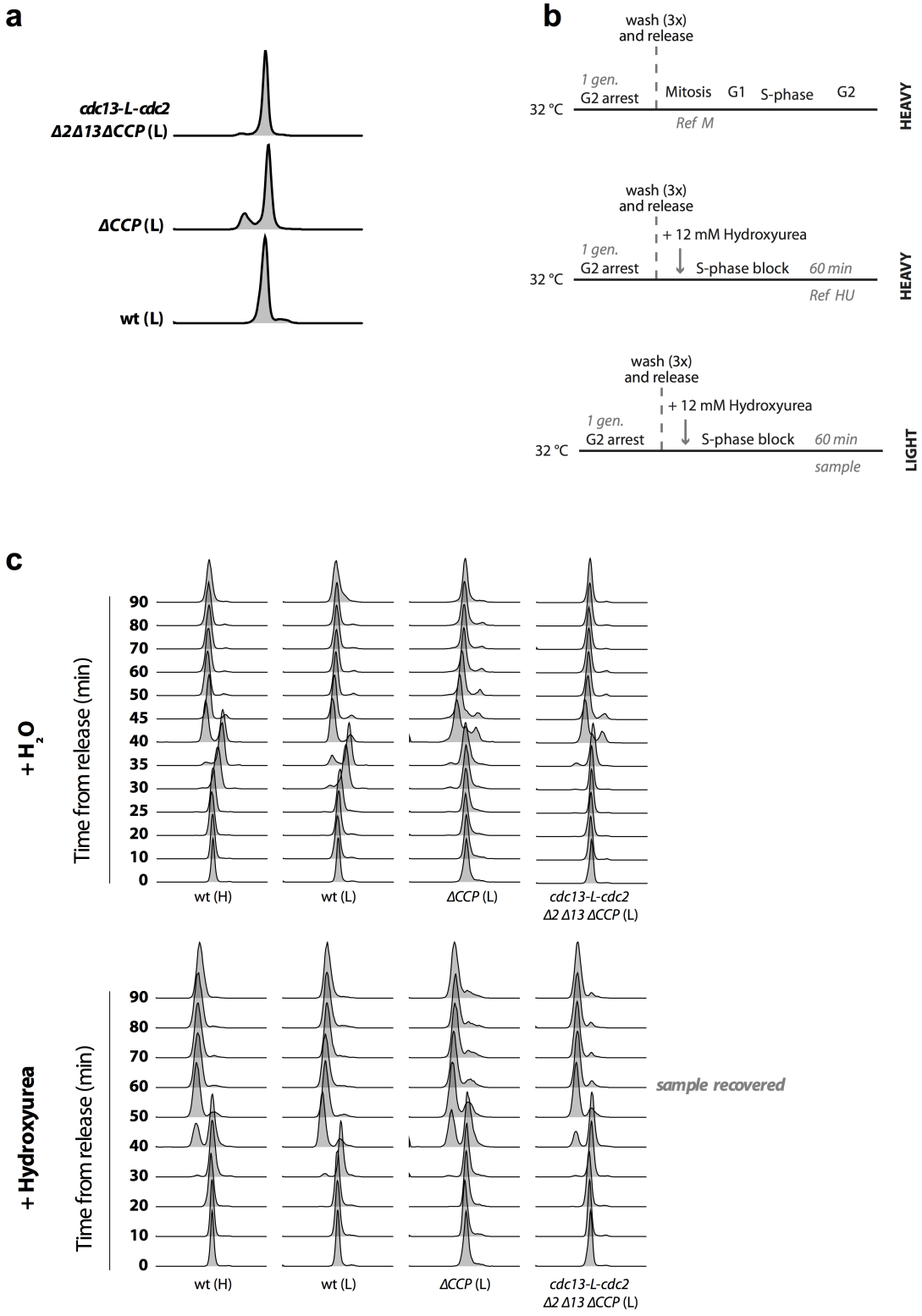

Figure 4.01 | Contribution of S-phase cyclins to the timing of S-phase
a DNA content during asynchronous exponential growth, determined by FACS, for wt (MS131) Δ*CCP* (MS200) and *cdc13-L-cdc2 Δ2 Δ13 ΔCCP* (MS213).
b schematic of experimental design. Unlabelled MS131, MS200 and MS213 were released from a G2 arrest and then Hydroxyurea (HU) or H_20 was added to all three cultures. Samples for proteomics were taken 60 min after release. Reference samples were taken from heavy labelled cultures in the same conditions (HU arrest) or released from a G2 arrest into mitosis.
c DNA content determined by FACS.

whilst Δ*CCP* and *cdc13-L-cdc2 Δ2 Δ13 ΔCCP* undergo bulk DNA synthesis 5-10 minutes later (35-50 min) (Figure 4.01c, upper panel) consistent with previous observations that Cig1 and Cig2 contribute to the timing of S-phase (Fisher and Nurse, 1996). The absence of a discrete 4C peak during S-phase for Δ*CCP* and *cdc13-L-cdc2 Δ2 Δ13 ΔCCP* is because the delay in DNA replication results in it coinciding with cell division, which splits the S-phase population either side of the 2C DNA content peak before S-phase has completed. When HU is added after the G2 arrest and release, cells arrest with a G1 DNA content (Figure 4.01c, lower panel). Protein extracts were recovered at 60 min, i.e. after the time at which cells would have completed S-phase if HU had not been added. Samples were mixed with one of two references as follows. Firstly, a heavy sample recovered in mitosis (i.e. cells 10 min after a G2 arrest and release), to allow the comparison with maximal phosphorylation. Secondly, an extracts from a heavy labelled wild type culture also arrested in HU to allow the quantification of the relative phosphorylation changes with and without S-phase cyclins.

4.2 The contribution of G1/S-phase cyclins to CDK substrate phosphorylation

Figure 4.02a shows the median phosphorylation of S-phase, biphasic and Mitotic CDK substrates, as well as the quantification of Cdc2-T167 phosphorylation in these three strains relative to maximal phosphorylation in mitosis. S-phase phosphorylation is greater than biphasic substrate phosphorylation, which is greater than mitotic substrate phosphorylation in all three backgrounds. This is consistent with cells arrested in S-phase being unable to attain sufficient CDK activity to majorly phosphorylate mitotic substrates. T167 phosphorylation is thought to occur after Cyclin-CDK complex formation (see Introduction, section 1.1.1) and as such can be use as a proxy for the amount of Cyclin-CDK complex formed. In the absence of S-phase cyclins there is significantly less T167 (~60%) suggesting that in these conditions Cyclin-CDK complex formation is limited by the availability of cyclin not CDK. In the wild-type background where CDK-complex formation has increased specifically due to Cig1, Cig2 and/or Puc1 there is a corresponding increase in S-phase substrate phosphorylation, but also in the

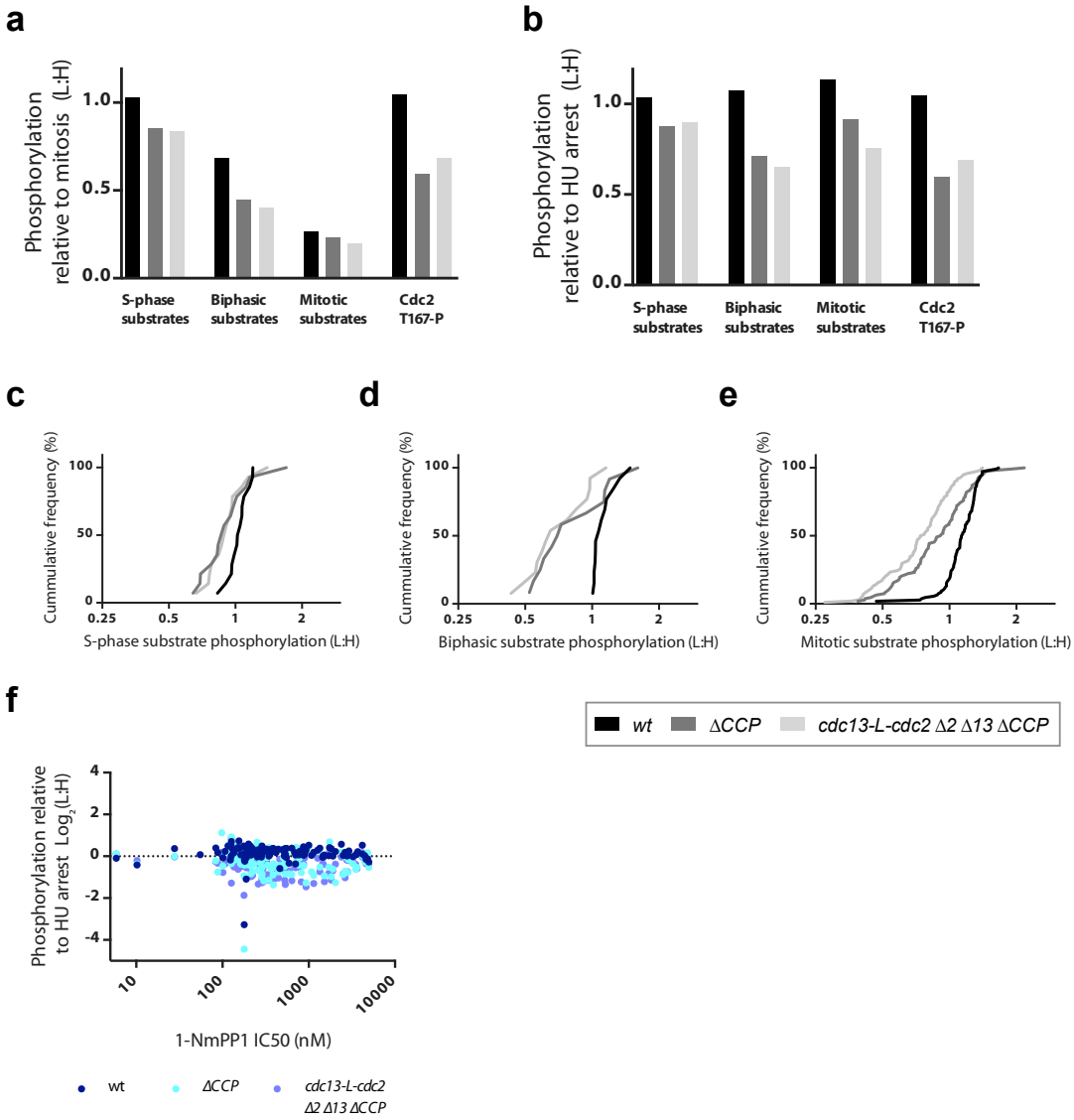

Figure 4.02 | Contribution of S-phase cyclins to CDK substrate phosphorylation
a&b Median phosphorylation of CDK substrates (S-phase, biphasic and mitotic) and the quantification of Cdc2-T167 phosphorylation, in wt (MS131) Δ*CCP* (MS200) and *cdc13-L-cdc2 Δ2 Δ13 ΔCCP* (MS213) when compared to a mitotic (**a**) or S-phase arrest (**b**) reference.
c-e The cumulative frequency of relative phosphorylation for CDK substrates (S-phase, biphasic and mitotic) in in wt (MS131) Δ*CCP* (MS200) and *cdc13-L-cdc2 Δ2 Δ13 ΔCCP* (MS213).
f Relative phosphorylation of CDK substrate sites plotted against each site sensitivity to CDK activity (i.e. IC50 to 1-NmPP1).

relative phosphorylation of biphasic substrates and mitotic substrates (Figure 4.02a). The increase in S-phase and biphasic substrate phosphorylation, as a proportion of maximal phosphorylation (i.e. L:H =1), is larger compared to mitotic substrates. This demonstrates that G1/S-phase cyclins contribute more to total phosphorylation of S-phase related substrates. This could be for one of two reasons. S-phase and biphasic substrates could be more sensitive to specific G1/S-phase cyclins and as such are more phosphorylated. Alternatively, S-phase

and biphasic substrates could be more responsive to the range of CDK activities in the cell to which S-phase cyclins are contributing in the HU arrest.

To attempt to distinguish between these two possibilities, the relative differences in S-phase, biphasic and mitotic phosphorylation was quantified when comparing these three samples to a heavy reference that was also arrested in HU. Figure 4.02b shows the median phosphorylation of the S-phase, biphasic and mitotic substrates in the wt, ∆*CCP* and *cdc13-L-Cdc2* ∆*2* ∆*13* ∆*CCP* background in an HU arrest compared to wt in an HU arrest. Figures 4.02c-e show the cumulative frequency of phosphorylation in each of the three backgrounds for each class of substrate. Both mitotic and S-phase substrate phosphorylation increases in the presence of Cig1, Cig2 and Puc1 by 10-15%. This suggests that when S-phase cyclins are associated with CDK, they activate the kinase but do not restrict the activity towards S-phase substrates. Mitotic substrates remain hypo-phosphorylated because CDK activity is too low to cause significant net mitotic substrate phosphorylation. Biphasic substrates show a greater difference with/without cyclins and are more sensitive to greater CDK activities than S-phase substrates but lower activities than mitotic substrates. The greater enhancement of biphasic substrate phosphorylation by Cig1, Cig2 and/or Puc1, could be because the presence of the S-phase cyclins tips total CDK activity over a third intermediate threshold for biphasic substrate phosphorylation or it could be because of cyclin specificity towards these substrates. Figure 4.02f shows the relative phosphorylation of CDK substrate sites in these three genetic backgrounds plotted against the site's sensitivity to CDK activity *in vivo* (i.e. 1-NmPP1 IC50). This suggests that the presence of S-phase cyclin, in these experimental conditions, increases the phosphorylation of substrates with the most intermediate sensitivity to CDK activity the most. This supports a model where the presence of Cig1, Cig2 and/or Puc1 simply increases the total CDK activity, and thus promotes the phosphorylation of substrate that are most sensitive to an increase in activity over this range.

If the amount of CDK substrate phosphorylation is used as a proxy for CDK activity the absence of S-phase cyclins seems to reduce CDK activity by ~15%. However, there is a >40% reduction in the amount of Cyclin-CDK complex formation (T167

phosphorylation). This indicates that Puc1, Cig1 and/or Cig2 do not contribute as much to total kinase activity per complex as Cdc13-Cdc2. Cd13-L-Cdc2 has a modest increase in T167 phosphorylation compared to ΔCCP. This could be because there is slightly more Cdc13 in the cell or the fact that the fusion between Cdc13 and Cdc2 promotes complex formation. Despite this, Cd13-L-Cdc2 is slightly less proficient at phosphorylating mitotic substrates than Cdc13 freely associating with Cdc2 (i.e. ΔCCP) (Figure 4.02a&b). This may be an artefact related to the fact that Cdc2 in the Δ*CCP* strain has an additional mutation to stabilise the F84G mutation, which is not necessary in the *cdc13-L-cdc2 Δ2 Δ13 ΔCCP* background.

4.3 Cig1, Cig2 or Puc1 unique phosphorylation events

If there where substrates that are truly unique to a S-phase cyclin(s) these would not have been defined as CDK substrates in Chapter 2, given that the definition of CDK substrates was done in the simplified CDK network background in which Cig1 Cig2 and Puc1 are absent. Figure 4.03 shows the quantification of all detected phosphorylation in the Δ*CCP* and *cdc13-L-Cdc2 Δ2 Δ13 ΔCCP* samples, relative to wt, plotted against each other. Putative Puc1, Cig1 and/or Cig2 unique substrates were defined as phosphorylation events that were fivefold less phosphorylated in in both Δ*CCP* and *cdc13-L-cdc2 Δ2 Δ13 ΔCCP* as compared to the heavy wt

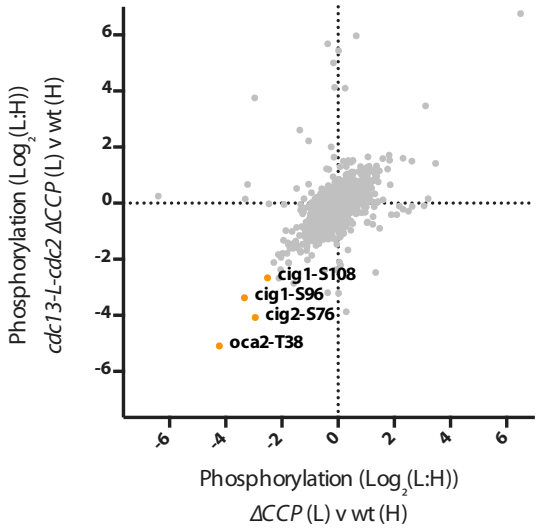

Figure 4.03 | S-phase cyclin unique phosphorylation events
Scatter plot of the quantification of all detected phosphorylation events (one point = one site) in Δ*CCP* and *cdc13-L-cdc2 Δ2 Δ13 ΔCCP* compared to wild-type when cells where arrested in S-phase with HU. Four sites (labelled yellow) had a >fivefold depletion in the absence of S-phase cyclins in both backgrounds.

reference. Only four phosphorylation events were more than fivefold depleted in ∆*CCP* and *cdc13-L-cdc2 ∆2 ∆13 ∆CCP* compared to wt: Oca2-T38, Cig1-S96, Cig1-S108, Cig2-S76. Oca2T38 is not at a T-P site and is also low in the null distribution (wt(L) : wt (H)) ruling it out as a possible G1/S-phase cyclin specific CDK substrate. The presence of substrates being phosphorylated exclusively by G1/S-phase Cyclin-CDK cannot be formally ruled out because phosphoproteomics coverage is not comprehensive. This analysis involves <5000 phospho-sites whilst all previously published phospho-proteomics studies have identified more than 18,000 phosphorylation events in *S. pombe* between them (Kettenbach et al., 2015). As such 18,000 can be considered a very conservative, but empirical, lower limit on the number of phosphorylation events *in vivo*. Despite this the detection of four sites on the G1/S cyclins that are significantly decreased serves as a good positive control and suggested if G1/S cyclin-unique substrates where prevalent they would have been detected here.

4.4 Discussion

These data demonstrate that S-phase cyclins can contribute to the phosphorylation of all types of CDK substrates and does not provide any convincing evidence that S-phase cyclins have dramatic specificity restricting them to early CDK substrates. Instead it points to a model where S-phase cyclins contribute to the profile of increasing CDK activity involved in the initiation of S-phase. The conclusion that S-phase cyclins contribute to the timing of S-phase, and do so predominantly by increasing CDK activity, as opposed to specificity, early in the cycle is consistent with the observation that *cig1*∆ *cig2*∆ cells initiate DNA replication after release from nitrogen starvation later than wild type cells (Fisher and Nurse, 1996). This delay corresponds to a delay in the accumulation of Cdc2 associated H1 kinase activity but not a significant delay in the rise in Cdc13 associated H1 kinase activity, indicating that Cig1 and Cig2 do not contribute to CDK activity indirectly upstream of Cdc13-Cdc2 activity (e.g. by regulating Cdc13-Cdc2 expression or activation) (Fisher and Nurse, 1996). The fact that Puc1, Cig1 and/or Cig2 binding to CDK does not increase CDK activity as much as Cdc13 binding is consistent with reports in *S. cerevisiae* that earlier cyclins have lower intrinsic CDK H1 kinase activity

(Loog and Morgan, 2005, Koivomagi et al., 2011b). A decrease in CDK activity per Cyclin-CDK complex could make it easier for rising CDK activity to resolve the S-phase activity threshold from the mitotic threshold by using a large number of less active complexes, as opposed to a smaller number of more active Cdc13-Cdc2 complexes. An alternative explanation for the data presented here is that Puc1, Cig1 and Cig2 are specific to S-phase substrates but by targeting CDK to them for phosphorylation a pool of Cdc13-Cdc2 is alleviated and able to now increase phosphorylation of mitotic substrates, such a mechanism would require S-phase substrates to function as competitive inhibitors of mitotic substrate phosphorylation as discussed in Chapter 3. The possible specificity of *S. pombe* S-phase cyclin should not be formally ruled out until cyclin specific CDK phosphorylation rates are quantified for a number of critical CDK substrates using different *S. pombe* Cyclin-CDK complexes.

Chapter 5. Beyond CDK: global phosphorylation dynamics of cell cycle kinases

In addition to CDK, multiple other kinases have been implicated in the processes of cell division and growth. The most notable examples involved in mitotic functions are the Polo-like kinases (Plk), Aurora Kinases and NIMA kinase. Fission yeast has a version of each: Plo1, Ark1 and Fin1 respectively. It is not obvious how and why mitotic phosphorylation is divided up between multiple regulatory kinases and a well resolved description of the phosphorylation dynamics downstream of these different kinases should further inform this question.

Plk1 is critical for bi-polar spindle assembly and is also involved in the auto-regulatory feedback loops that control CDK activity at mitotic entry, the regulation of chromosome cohesion and cytokinesis (Archambault and Glover, 2009). *S. pombe* Plo1 is necessary for bi-polar spindle formation and promotes septation (Mulvihill et al., 1999, Tanaka et al., 2001). Plo1 localises to the SPB early in mitosis and to the site of septation later (Mulvihill et al., 1999, Bahler et al., 1998a). Plo1's localisation to the SPB as well as its association with *bona fide* substrates such as Mid1 requires its Polo-box domain (PBD) (Reynolds and Ohkura, 2003, Almonacid et al., 2011). Higher eukaryotic Aurora B is part of the chromosome passenger complex (CPC) which promotes chromosome condensation, appropriate kinetochore microtubule attachment and spindle mid-zone formation, whilst also antagonising chromosome sister chromatid cohesion and regulating some aspects of cytokinesis (Vader and Lens, 2008, Carmena et al., 2012). The only *S. pombe* Aurora kinase, Ark1, concentrates at the kinetochore during early mitosis and distributes along the spindle during anaphase (Petersen et al., 2001). Ark1 is required for chromosome condensation, segregation and spindle assembly checkpoint function (Leverson et al., 2002, Petersen and Hagan, 2003) and Ark1 phosphorylation of the kleisin condensin subunit, Cnd2, promotes condensin recruitment to the chromosomes (Nakazawa et al., 2011, Tada et al., 2011). NIMA kinase was first identified as a protein essential for mitotic commitment in the filamentous fungus *Aspergillus nidulans* (Morris, 1975). The *S. pombe* ortholog is important for chromosome condensation and spindle assembly as well as participating in the activation and

recruitment of Plo1 to the SPB (Grallert et al., 2013a, Grallert and Hagan, 2002, Krien et al., 1998, Krien et al., 2002).

A combination of biochemical and phospho-proteomics approaches have been used to define phosphorylation consensus sequences for these kinases, across eukaryotic systems (Koch et al., 2011, Cheeseman et al., 2002, Nakajima et al., 2003, Grosstessner-Hain et al., 2011). Positional scanning oriented peptide library screening has corroborated these consensus sites and highlights the fact that human Plk1, Aurora A&B and Nek2 (NIMA-related kinase) have a strong preference for any residue besides Proline in the +1 position. This means that the CDK consensus site is mutually exclusive with that of other mitotic kinases. This may form the basics of a “division of labour” between CDK and other mitotic kinases, despite the overlap in their spatial distributions during mitosis (Alexander et al., 2011).

In this chapter I present an analysis of cell cycle dependent phosphorylation (quantified in Chapter 2), besides that directly mediated by CDK (Cdc2). This analysis identifies a number of trends that seem to allow the amplification and diversification of the cell cycle dependent phosphorylation beyond CDK signalling. It also hints at possible mechanisms by which cell cycle kinase cascades are coordinated.

5.1 Global phosphorylation is highly dynamic during the cell cycle

The global behaviours of the phosphoproteome were analysed during two sequential cell cycles to investigate the contributions of, and the connectivity between, different kinases over the cell cycle. Cells were synchronised after release from a G2 arrest imposed by chemical inhibition of Cdc2 (by 1 μM 1-NmPP1) (Figure 2.08). Compared to previous cell cycle phosphoproteomics studies this analysis involves significantly more time points (20) and also analyses two sequential synchronous divisions (Olsen et al., 2010, Carpy et al., 2014). The ability to define behaviours that are reproduced over two sequential divisions is important as it allows the discrimination of cell cycle dependent phenomena from possible artefacts of cell cycle perturbation used for synchronisation, that would be seen only during the first cycle after release, such as the temperature shifts to synchronise cell in previous *S. pombe* proteomics studies (Bicho et al., 2010, Carpy et al., 2014).

5.1.1 Global phosphorylation is highly dynamic and synchronised during the cell cycle

Hierarchal clustering and principal component analysis (PCA) were used to assess the global changes in, and variance between, the phosphoproteome and proteome across the synchronised cycles. PCA is a powerful statistical approach used to describe the proximity of different conditions (i.e. time points) within a multi-dimensional dataset (i.e. thousands of different phosphorylation events). It defines the first (principal) component as an orthogonal coordinate axis that describes the direction of the maximal variance between all samples through multi-dimensional space. It then defines a second component that accounts for the next most maximal variance, orthogonal to the first component, and so on. The relative distance between any two conditions on a component reflects the relative differences between those two samples, in the direction for which that axis accounts for variance. PCA of the phosphoproteome clusters samples from the same cell cycle stages demonstrating that the phosphoproteome is less different

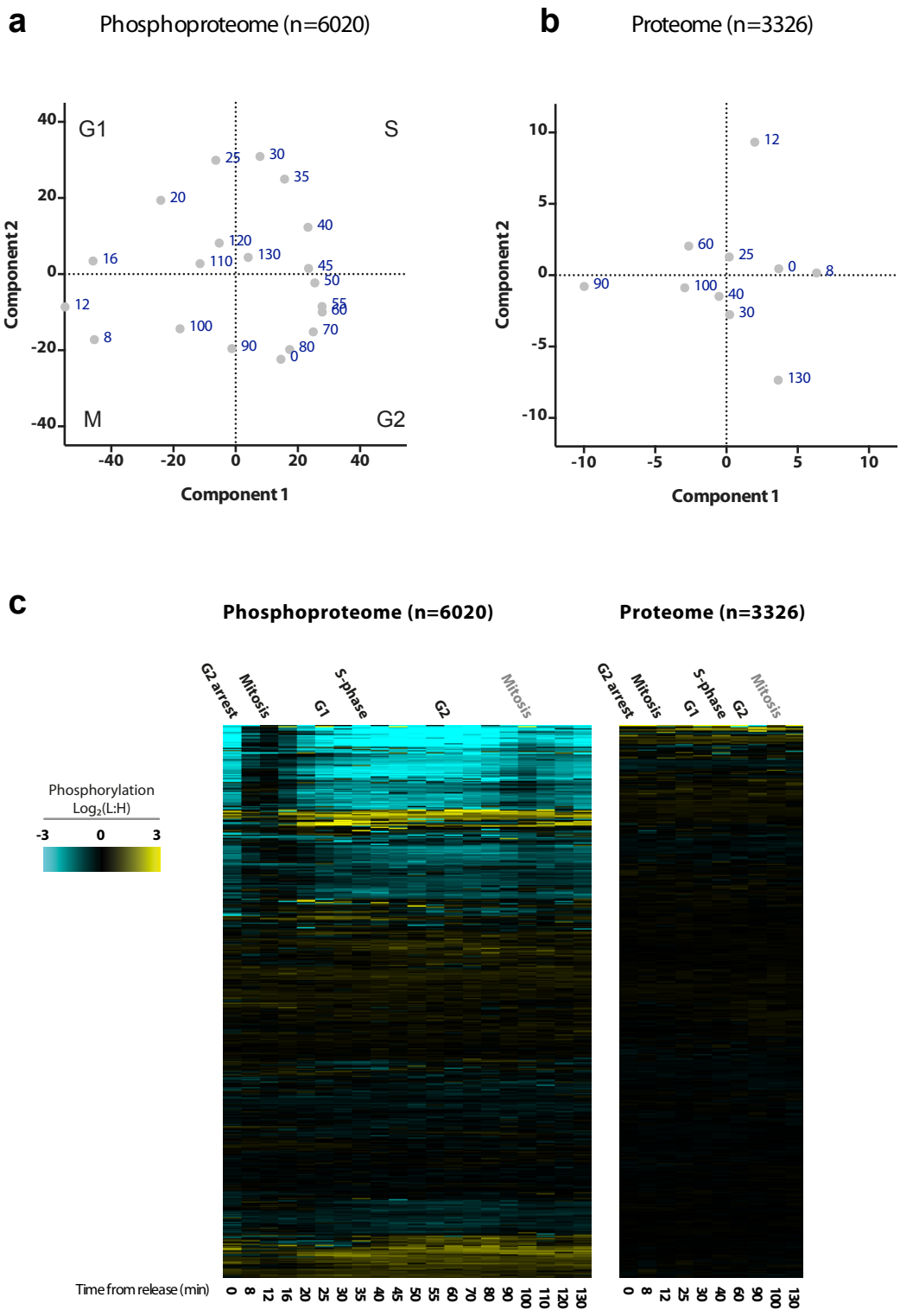

Figure 5.01 | Global dynamics and synchrony of the phosphoproteome and proteome during the cell cycle

a&b Plot of component one and two from principle component analysis of the phosphoproteome and proteome. Each point corresponds to a single time point during over two sequential cell cycles. Points closer together are more similar in the respective component.

c Heat map of total phosphorylation and protein levels, after hierarchical clustering by Euclidian distance. Each row corresponds to a single phosphorylation event or protein and each column corresponds to a different time point after release from a G2 arrest. Values outside the display range ($3 > Log_2(L:H) > -3$) are set to the closest extreme.

between samples taken from the same cell cycle stage (Figure 5.01a). The first component accounts for 35.1% of the variance whilst the second component accounts for 14.5% of the variance. In the second division cycle (90-130 min) the variance between samples is less, due to the reduced synchrony of the culture which is why time points spiral toward the origin on the plot of component 1 v component 2. The largest differences (component 1) in the phosphoproteome occur between interphase and mitosis. This is not true for the proteome, for which PCA is unable to draw out differences between time points that cluster cell cycle states together (Figure5.01b). This demonstrates a global synchrony to the phosphoproteome that corresponds to cell cycle progression, but is absent at the level of the proteome. The degree and direction of global change in phosphorylation and protein can be seen in Figure 5.01c, a heat map of all phosphosites relative phosphorylation values after hierarchical clustering. This shows a wide dynamic range in the changes in phosphorylation during the cell cycle with a large number of sites peaking in mitosis, whilst the proteome is broadly unchanged. 33.7% of the phosphosites in Figure5.01c show a threefold change during the cell cycle.

5.1.2 Defining dynamic phosphorylation at non-CDK substrate sites

To dissect the contributions to specific behaviours all subsequent analysis was restricted to singly phosphorylated peptides. This should eliminate artefacts where quantification of phosphorylation at a particular site is influenced by changes in phosphorylation of adjacent sites on the same peptide. As discussed in Chapter 2, hundreds of S/T-P sites are regulated by CDK, and 42.0% (629/1497) of the S/T-P on singly phosphorylated peptides change more than threefold across the cell cycle, whilst 24.3% (685/2814) of sites not at a S/T-P sites changed similarly. Sites that change threefold were considered to be dynamic. The consensus motifs of mitotic cell cycle kinases (Sheet03) were analysed. This shows that dynamic S/T-P sites are enriched in the CDK consensus site (S/T-P-X-K/R). In contrast dynamic non-S/T-P are enriched at the Plk1 sequence consensus motifs and at Ark1-dependent phosphorylation sites, previously identified in a phospho-proteomics screen (Koch et al., 2011). They are also enriched on proteins phosphorylated by CDK at other

residues (defined in Chapter 2) suggesting co-regulation of substrates between CDK and other kinases is commonplace.

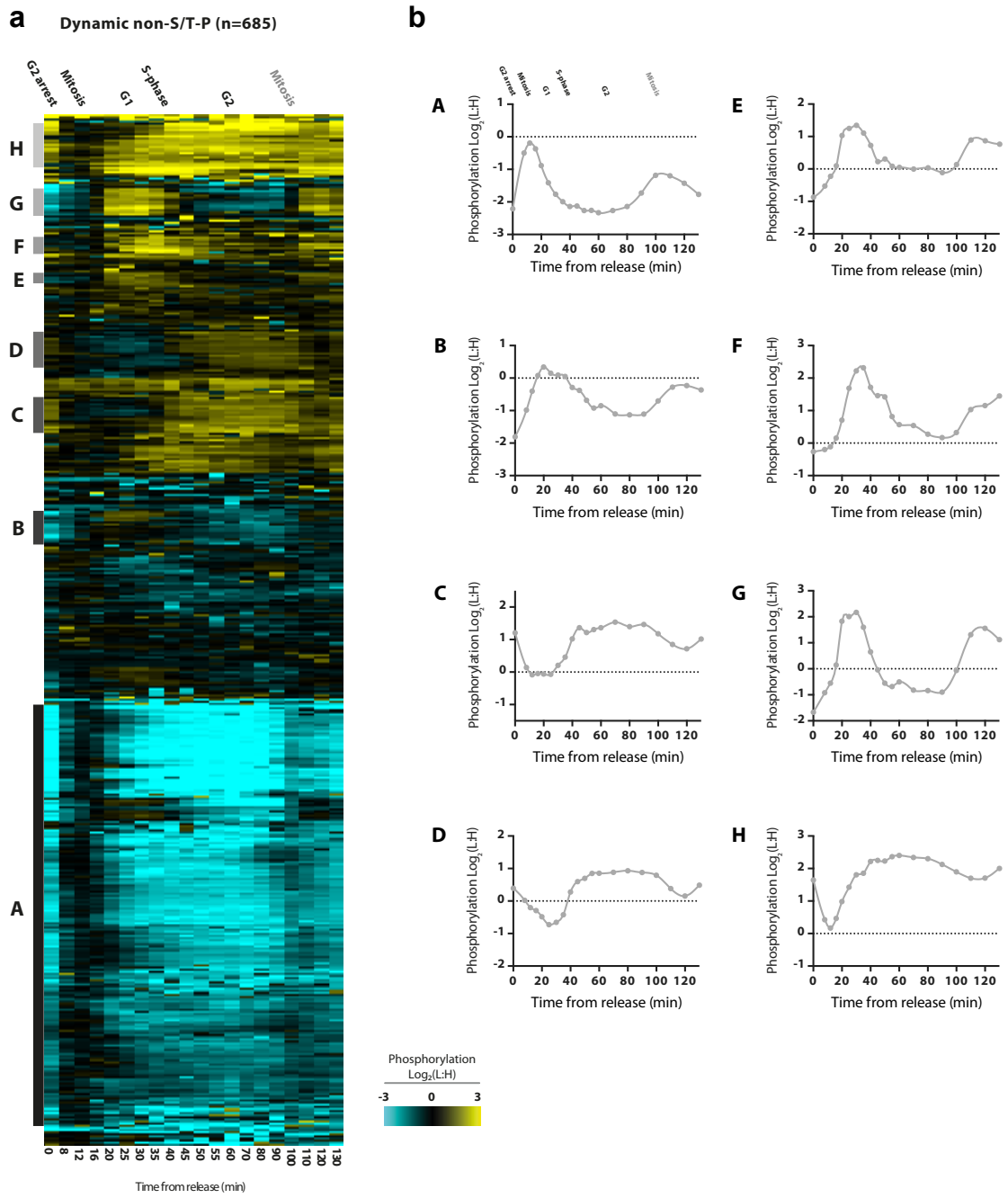

Figure 5.02 | Dynamics of non-CDK substrate phosphorylation during the cell cycle
a Heat map of phosphorylation after release from G2 arrest, of all non S/T-P sites that changed more than threefold during the cell cycle. Each row corresponds to a single phosphorylation event and each column corresponds to a different time point after releaser from a G2 arrest. Rows are ordered by hierarchical clustering according to Euclidian distance. Different clusters (A-H) represent a number of cell cycle dependent behaviours that are consistent over the two sequential cell cycles.
b Median phosphorylation of all sites in clusters A-H, after release from a G2 arrest.

To get a sense of the differing outputs of the kinases besides CDK, these 685 dynamic non S/T-P sites were hierarchically clustered by Euclidean distance and a number of discrete behaviours, which showed consistent changes over the two cell cycles, were defined in cluster A-H (Figure 5.02a). Figure 5.02b shows the median profile for all sites in a given cluster and Figure 5.03 shows the distribution of amino acids around the phosphorylation site for all phosphorylation events in each cluster.

Cluster A defines a significant portion of the phosphoproteome that increases during mitosis. These mitotic sites are enriched in Ark1 substrate sites described by Koche et al. (2011) as well as all three Polo-like kinase (Plk1) consensus motifs. Cluster A is the only cluster enriched on proteins with CDK-dependent phosphorylation events at S/T-P residues elsewhere in the protein. Cluster A is also enriched in cell cycle related GO-terms such as chromosome segregation, cell division and the kinetochore. Cluster B phosphorylation peaks later in mitosis and is sustained until S-phase/cytokinesis when these sites are dephosphorylated until the next mitosis. Cluster B was also enriched in previously defined Ark1 dependent phosphorylation sites as well as sites on proteins involved in chromosome organisation.

A smaller number of sites do not peak in mitosis but still have a dynamic and consistent behaviour over the two cell cycles (clusters C-H) (Figure 5.02). Of these, clusters C and D both decline during nuclear and cell division, when the majority of

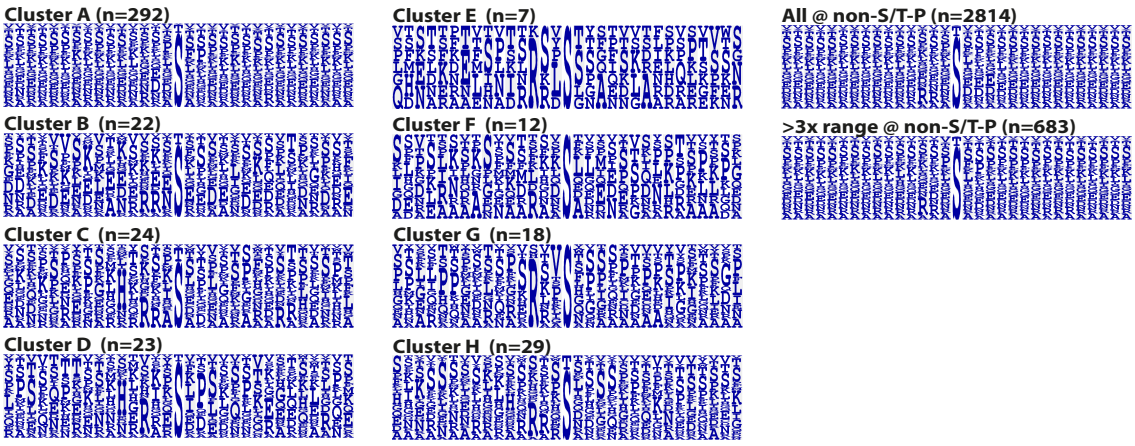

Figure 5.03 | Phosphorylation site consensus sequence
Amino acid distribution around the phosphorylation site for all phosphorylation sites in clusters A to H, as well as all non-S/T-P phosphorylation sites and dynamic non S/T-P phosphorylation.

the dynamic phosphoproteome is maximally phosphorylated, peak during G2 and decline as cells enter the second mitosis. The sequences, surrounding these phosphorylation sites in cluster C&D, have a significant enrichment of a -5 histidine and -3 arginine (Figure 5.03). In terms of protein function, cluster D is enriched in proteins annotated to secretion and lipid metabolism gene ontologies. Clusters E and G show the opposite behaviour and peak during G1/S, are low through the rest of G2, and start to rise again at the end of the next mitosis. Both of these clusters are heavily enriched for a -3 arginine with respect to the phosphorylation site. All dynamic non-S/TP sites, clusters A-H, and the enrichments are listed in supplementary Sheet03.

5.2 Mitotic Kinase substrate phosphorylation and their dependency on CDK activity

The evolution of more than one kinases to control the progression through mitosis may have been driven by the need for different substrates to be phosphorylated with precisely resolved differences in timings/dynamics, i.e. diversifying the range of substrate phosphorylation profiles. Because there has been no systematic analysis of all mitotic kinase's targets in *S. pombe*, putative Ark1 (Aurora), Plo1 (Plk) and Fin1 (NIMA kinase) substrates were defined in the dataset and were compared to the phosphorylation dynamics of CDK substrates. Although putative substrates are not direct substrates, the average behaviour of their phosphorylation should reflect the collective dynamics of true substrates.

Putative Ark1, Plo1 and Fin1 substrates were defined using the dataset of 685 dynamic phosphorylation events (quantified only from singly phosphorylated peptides). This dataset contained 84 Aurora, 114 Plk and 54 NIMA kinase consensus sites (consensus sequences listed in Sheet03) and 30 Aurora, 80 Plk and 26 NIMA kinase consensus sites that fall with in the mitotic clusters (A&B). Given these three kinases are known to function during mitosis these mitotic sites were defined as putative substrates. Examples of these sites are shown in Figure 5.04a-c. Putative Ark1 substrate sites are enriched in previously describe Ark1 dependent phosphorylation events whilst Fin1 and Plo1 sites are enriched on proteins phosphorylated by CDK at other residues, suggesting a significant amount of cross talk may exist between these kinases and Cdc2. The examples in

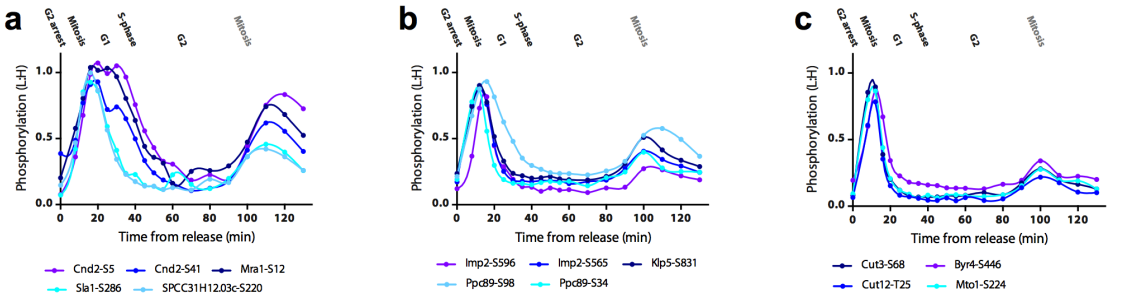

Figure 5.04 | Mitotic kinases: putative Ark1, Plo1 and Fin1 kinase substrate phosphorylation during the cell cycle
a-c Examples of the relative phosphorylation (after smoothing) of Ark1 and putative Plo1 and Fin1 substrates after release from a G2 arrest (A smoothed line, Spline, connects values).

figure 5.04a-c show that Ark1 substrates peak at the end of mitosis and during G1 whilst putative Plo1 and Fin1 substrates peak in mitosis, and that Plo1 phosphorylation persist slightly longer than Fin1 substrate phosphorylation.

As an approximation of the phosphorylation output of these kinase the median phosphorylation of Cdc2, Ark1 and putative Plo1 and Fin1 substrates during the first mitotic exit and second mitotic entry are shown in Figure 5.05a&b. The first mitotic exit was analysed because it is the most synchronous. The second mitotic entry was analysed as it is more physiological than the abrupt release from Cdc2 inhibition after G2 arrest at the first mitotic entry. Putative Fin1 and Plo1 substrates are as described above, Cdc2 substrates were defined in Chapter 2 and Ark1 substrates are defined as the overlap between putative Ark1 substrates and Ark1 dependent phosphorylation sites defined by Koch et al. (2011). During mitotic exit the substrates of different mitotic kinase are dephosphorylated in sequential waves with Cdc2 substrates dephosphorylated first, followed almost immediately by Fin1 substrates then Plo1 and finally Ark1 substrates. During mitotic entry Cdc2 substrates are phosphorylated first followed by Fin1 then Plo1 substrates. Ark1 substrate phosphorylation peaks last, about ten minutes after Cdc2 substrate phosphorylation. These timing intervals may represent an important aspect of the ordering of the temporal progression through mitosis and the timely completion of cytokinesis. There are also differences between putative substrates of the same kinase, for example some of the Ark1 substrate sites peak at the beginning of G1 and then immediately start to be dephosphorylated, whilst phosphorylation on others substrates, such as the non-SMC condensin subunit Cnd2, only start to be removed in the middle of S-phase/cytokinesis (figure 5.04c).

Cells arrested in G2 by Cdc2 inhibition (1 μM 1-NmPP1) do not have Ark1, Plk1 or Fin1 substrate phosphorylation until the 1-NmPP1 is washed out, indicating that the phosphorylation of these kinases' substrate is down-stream of Cdc2 either directly, or indirectly in response to the initiation of mitotic events. To asses these dependencies, the phosphorylation of these substrates was analysed after complete chemical inhibition of Cdc2 during mitosis (10 μM 1-NmPP1). Figure 5.05c shows the median relative phosphorylation of Cdc2 and Ark1 substrates and

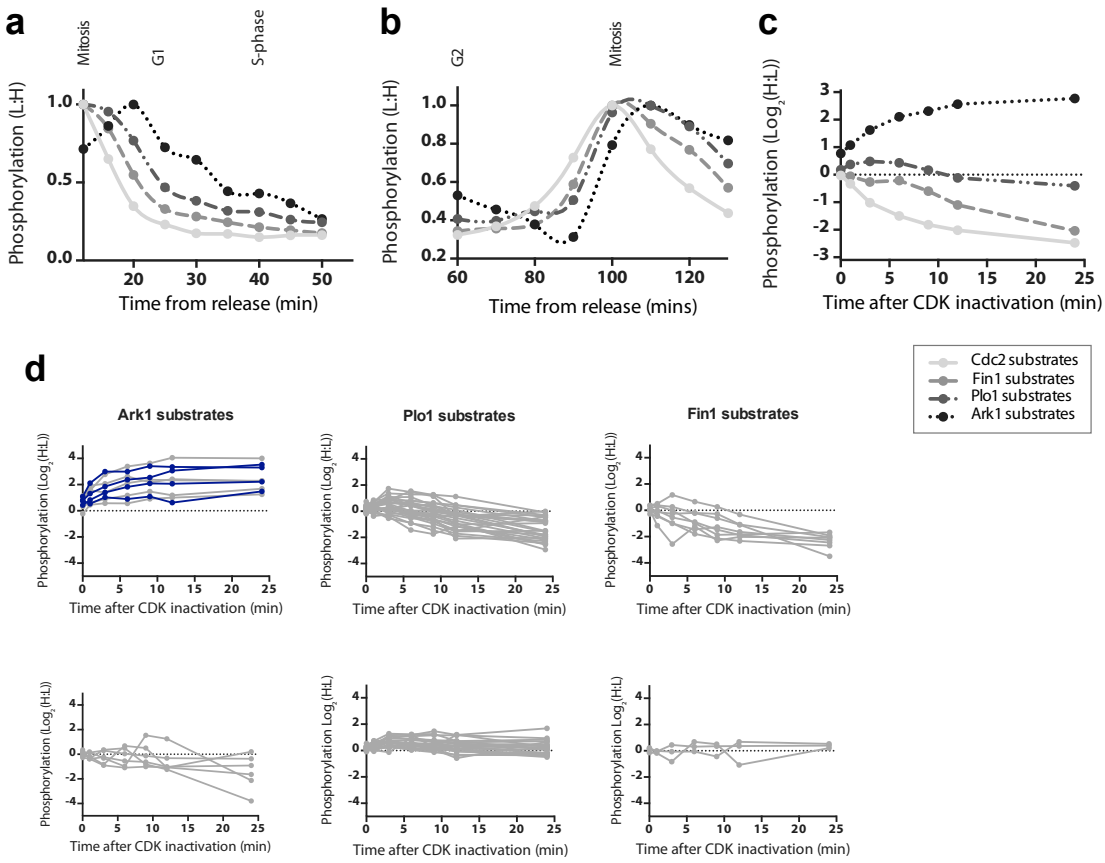

Figure 5.05 | Ark1, Plo1 and Fin1 substrate phosphorylation dependencies on CDK activity
a-c Median phosphorylation of Akr1 and Cdc2 substrate sites and putative Plo1 and Fin1 substrate sites during (**a**) mitotic exit, (**b**) mitotic entry or (**c**) after CDK inactivation in mitosis with 10 μM 1-NmPP1.
d Relative phosphorylation of all putative Ark1, Plo1 and Fin1 substrates after CDK inactivation in mitosis (see Figure 2.02a for experimental design. Putative substrate sites were split across two graphs (upper and lower panel) showing two distinct behaviours. Ark1 dependent phosphorylation events defined by Koch et al. (2011) are shown in blue. One putative Fin1 site is not shown as it falls outside the axis limit.

putative Plo1 and Fin1 substrates after Cdc2 inhibition, whilst Figure 5.05d shows individual profiles of putative Ark1, Plo1 and Fin1 kinase substrates after Cdc2 inhibition. Cdc2 substrates start to be dephosphorylated immediately after Cdc2 inhibition whilst putative Fin1 substrates show a small delay of 3-6 min before they start to be dephosphorylated. Plo1 substrates rise slightly or are stable up to approximately 6 min after Cdc2 inhibition, when the majority start to be dephosphorylated. In contrast putative Ark1 substrate phosphorylation is refractory to CDK inhibition and is either stable (Figure 5.05d lower panel) or continues to increase (Figure 5.05d upper panel) in phosphorylation, even after Cdc2 has been inhibited for 24 min.

The dependencies of these kinases on Cdc2 activity suggest that Cdc2 may directly positively regulate Fin1 and Plo1 activity, whereas Ark1 does not require the continued presence of Cdc2 activity, once mitosis is initiated. These differential dependencies on Cdc2 would be sufficient to explain the progressive ordering of phosphorylation during mitotic entry and exit. Cdc2 activation results in the phosphorylation of its substrates that then initiates the activation of Fin1 and Plo1 substrate phosphorylation, which rise shortly after Cdc2 substrate phosphorylation in mitosis.

When Cdc2 is inactivated, or when cells progress through mitotic exit, putative Fin1 substrate are dephosphorylated faster than those of Plo1, suggesting that Plo1 activity or substrate phosphorylation is more stable than that of Fin1. This may be because Plo1 substrates are less sensitive to phosphatases during mitotic exit than Fin1 substrates or that Plo1 activation is multiple steps downstream of Cdc2, generating a time lag in the response. There are multiple possible mechanisms, which are not mutually exclusive, by which Plo1 substrate phosphorylation could be dependent on Cdc2 activity. Cdc2 phosphorylates Plo1 and other SPB proteins that may serve to activate Plo1. So when CDK is inactivated Plo1 or its activators would then become dephosphorylated, reducing the rate of Plo1 substrate phosphorylation which in turn allows their net dephosphorylation. Plo1 is also a putative substrate of itself, suggesting that auto-phosphorylation may play a role in this process. Alternatively, Cdc2 may phosphorylate Plo1 substrates priming them for subsequent Plo1 phosphorylation as has been described for a number Plk1 substrates in higher eukaryotes (see Introduction, section 1.2.3), consistent with the fact that putative Plo1 substrates are enriched on CDK substrates. One putative Fin1 substrate is Cut12 (T25) a SPB protein that has been characterised as a *bona fide* Fin1 and Cdc2 substrate, the phosphorylation of which is important for the recruitment and activation of Plo1 at the SPB3 (Grallert et al., 2013a). As such it is possible that part of the dependency of Plo1 on Cdc2 works via Fin1 phosphorylation of substrates such as Cut12. Cut12 and most of the putative Fin1 substrates are also Cdc2 substrates suggesting that Fin1 may work to immediately amplify the effects of Cdc2 phosphorylation on a number of Cdc2 substrates.

In contrast Ark1 substrate site phosphorylation is refractory to Cdc2 inhibition suggesting that once mitosis is initiated Ark1 activity is independent of Cdc2 activity. This allows Ark1 phosphorylation to persist long after mitotic exit until the point of S-phase/cytokinesis. Consistent with this Cdn2 (a major Ark1 substrates) function is required throughout mitosis as well for a period after anaphase completion (Nakazawa et al., 2011). As mentioned above different Ark1 substrates are dephosphorylated at different times as cells progress from mitosis to the end of cytokinesis. This could be due to different phosphatases, the activities of which are temporally regulated during mitotic exit, targeting different Ark1 substrates for dephosphorylation (Grallert et al., 2015).

5.3 Regulation of the NDR kinases, Sid2 and Orb6, as revealed by the dynamics of substrate phosphorylation

In addition to the classic mitotic kinases described above, the Septum initiation network (SIN) ensures the appropriate coordination between cell division and nuclear division. SIN redundantly contributes to the assembly of the actomyosin ring and is essential for the maturation, constriction and disassembly of the cytokinetic ring (Pollard and Wu, 2010). The SIN pathway's terminal output is Sid2 kinase activity. A number of Sid2 substrates have been described including Clp1, Cdc11, Cdc12 and Klp2 (Bohnert et al., 2013, Chen et al., 2008, Feoktistova et al., 2012, Mana-Capelli et al., 2012). Some of these were also identified in a proteomics screen for Sid2 substrates which identified the following proteins: Sog2, Nak1, Clp1 Rgf1, Ase1, Pos1, Ppk25, Mph1, Ipp1, Scw1, Cdc11 and Mug72 (Gupta et al., 2013). Sid2 preferentially phosphorylates the sequence: R-X-X-S and Sid2 activity is thought to start to rise in anaphase (Chen et al., 2008, Feoktistova et al., 2012). Figure 5.06a shows all detected phosphorylated R-X-X-S motifs on the above proteins that increase during in mitosis (i.e. cluster A&B). These sites were defined as Sid2 substrate sites. Interestingly these phosphorylation sites fall into two categories: those that peak in mitosis and those that increase during mitosis but continue to rise in phosphorylation until cytokinesis. These two behaviours mirror the behaviour of four phosphorylation sites in the N-terminal domain of Sid2 itself. Two sites at the minimal CDK consensus (S42 & S86) peak in mitosis, one of which is a *bona fide* CDK substrate site (S86) (Chapter 2 & Sheet02). S56 and S99 rise during mitosis, peak during G1 and decline after cytokinesis. These two sites are 13/14 amino acids C-terminal to one of the S/T-P sites, suggesting there may be some importance to the local arrangement of phosphorylation sites of different kinases that phosphorylate Sid2, possibly involving a "hand-over" event in the activation of Sid2 from CDK to another kinase that targets proximate but distinct sites. This dual regulation may reflect or contribute to the change in phosphorylation of Sid2 substrates.

Cluster E and G are enriched in R-X-X-S sites and phosphorylation peak during cytokinesis (Figure 5.02 & 5.03), but unlike the Sid2 substrates described above,

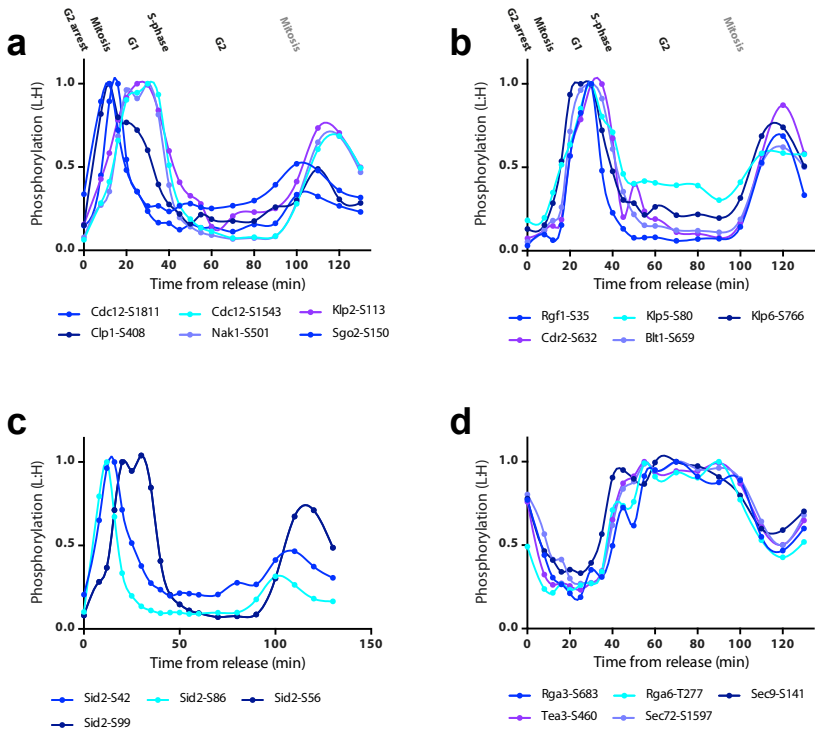

Figure 5.06 | Sid2 substrates and putative Orb6 substrates involved in polarised growth **a-d** examples of the relative phosphorylation (after smoothing and normalisation to maximum phosphorylation value) of (**a**) sid2 substrate phosphorylation, (**b**) sid2 phosphorylation (**c**) G1/septation phosphorylation at R-X-X-S sites and (**d**) putative Orb6 phosphorylation (at H-X-X-X-X-S/T, peaks in G2). (A smoothed line, Spline, connects values).

phosphorylation sites in cluster E and G do not increase in phosphorylation during mitosis. Some of these proteins (Figure 5.06b) are involved in process related to cytokinesis, for example Blt1 and Cdr2 are cortical node proteins that contribute to septum positioning. Cdr2 transiently dissociates from cortical nodes towards the end of mitosis: a possible function for this phosphorylation (Pollard and Wu, 2010). Klp5 and Klp6 are also in these clusters and contribute to the cytokinetic actomyosin ring contractility. However, none of the R-X-X-S phosphorylation events in cluster E or G are on proteins previously defined as Sid2 substrates by Gupta et al. (2013) suggesting they may be the substrates of other kinases that peak during G1/S. For instance the Cmk1 transcript and Cmk2 protein levels have been reported to peak during G1/S and are both orthologs of Calmodulin activated kinases that phosphorylate a minimal consensus site of R-X-X-S/T (Swulius and Waxham, 2008, Alemany et al., 2002, Rasmussen, 2000).

During mitosis and cytokinesis cells arrest polarised growth (Mitchison and Nurse, 1985) and Sid2 phosphorylation of Nak1, resulting in inactivation of the NDR-related kinase Orb6, is thought to contribute to this arrest (Gupta and McCollum, 2011, Ray et al., 2010). Nak1-S501 phosphorylation rises during mitosis and peaks just before cell division (Figure 5.06a). Orb6 organises the polarisation of the actin

patches to cell ends via the spatial regulation of Cdc42 activity, but to date no direct Orb6 substrates have been described (Das et al., 2009, Verde et al., 1998). The NDR kinase consensus motif is enriched in a -5 histidine and a -3 arginine or lysine (Hao et al., 2008). Interestingly Cluster C and D are enriched in both of these traits indicating that phosphoproteins in these clusters could represent direct Orb6 substrates (Figure 5.03). The phosphorylation of NDR consensus sites in Cluster C and D show the inverse phosphorylation pattern to that of Sid2 substrates and are phosphorylated throughout G2. This is consistent with the hypothesis that when cytokinesis is completed Sid2 is inactivated, resulting in Nak1 dephosphorylation permitting Orb6 activation and Orb6 substrate phosphorylation and thus re-initiate polarised growth (Figure 5.06d). Consistent with Orb6's role in polarised growth cluster C and D include the polarity factor Tea3 (Arellano et al., 2002) and are enriched in proteins involved in lipid transport and exocytosis (e.g. Sec72, Sec9 & Oxysterol-binding protein ortholog SPBC2F12.05c). The timing of phosphorylation and annotated functions of these phosphoproteins supports the conclusion that a number of these substrates may indeed be direct Orb6 substrates.

5.4 Discussion

CDK seems to be responsible for an impressively large proportion of cell cycle regulated phosphorylation. However, a significant subset of non CDK consensus sites (non S/T-P) are also dynamic during the cell cycle. These phosphorylation events coordinate the execution of cell cycle processes, downstream of a number of cell cycle kinases besides CDK. Here five other kinases have been investigated: NIMA kinase, Polo-like kinase, Aurora Kinase, and two NDR kinases involved in the septation initiation network (Sid2) and the MOR pathway (Orb6). Analysing *bona fide* and putative substrate of these kinases reveals multiple sequential waves of phosphorylation that proceed as cells undertake the program of cell division. This phosphorylation is inversely related to interphase phosphorylation implicated in cell growth, possibly downstream of the Orb6 kinase.

The crosstalk and connectivity between these kinases is striking and functions at two levels: the regulation of each other as well as the phosphorylation of common substrates. This diversity of kinases seems to allow the amplification and diversification (e.g. via persistence after CDK inactivation) of CDK signalling during mitosis. CDK substrate phosphorylation peaks early in mitosis and starts to decline once anaphase is initiated and Cyclin degradation starts. The phosphorylation of substrates, by other mitotic kinases, peaks in mitosis but have different phosphorylation dynamics during mitosis to CDK substrates. For instance, the phosphorylation of putative substrates of Fin1, Plo1 Ark1 and Sid2 increases after CDK activity rises at mitotic entry. Most of these sites are then dephosphorylated after Cdc2 inactivation at mitotic exit in sequential waves, in a manner corresponding to the respective kinase's dependency on CDK activity. The altered profiles and persistence of other mitotic kinases downstream of Cdc2 allows the diversification of CDK signalling via these kinases.

Furthermore, some kinase (e.g. Ark1 and Sid2) may have substrates with significantly different phosphorylation profiles suggesting that the progression through mitosis and cytokinesis may involve shifting exposure to, or specificity for, certain substrates.

Chapter 6. General discussion

The main conclusions of this thesis are as follows. There are hundreds of CDK substrates in *S. pombe* that are first phosphorylated by CDK at different times during the cell cycle. The early phosphorylation of S-phase substrates persists and is not reversed during G2 and mitosis. Mitotic substrates are phosphorylated at mitotic entry, and both S-phase and mitotic substrates are dephosphorylated at mitotic exit. There is also a third intermediate category in which substrate phosphorylation increases significantly at both transitions, and peaks at G2/M (biphasic substrates). The differences between these categories of substrates can be accounted for by differences in their *in vivo* sensitivity to CDK activity: S-phase substrates being more sensitive than biphasic, which are in turn more sensitive than mitotic substrates. This supports the notion that rising CDK activity orders cell cycle events via the attainment of substrate specific CDK activity thresholds, with low activity resulting in S-phase substrate phosphorylation, and thus initiating DNA replication, and higher activity driving mitotic entry via the phosphorylation of mitotic substrates. Furthermore, when the order of CDK substrate phosphorylation is perturbed, there is a corresponding reordering of the cell cycle, supporting the conclusion that activity dependent differential phosphorylation of CDK substrates not only correlates with cell cycle events but is rate-limiting for the initiation of the respective cell cycle transition.

Of those proteins phosphorylated in S-phase, there are a number of substrates previously described as involved in origin licensing (e.g. Orc1 and Orc2) and others involved in origin firing (e.g. Drc1 and Sld3) (see Introduction, section 1.2.1). Both become initially phosphorylated during the same window at G1/S, consistent with the notion that the inhibition of origin licensing has to occur at the same time as, or before, the initiation of origin firing to prevent fired origins from re-licensing and re-firing. However, phosphorylation on Orc1 and Orc2 continues to increase significantly during G2/M, in contrast to Drc1 or Sld3. The significance of this is unclear, but it may reflect a preference to initially phosphorylate ORC proteins that are in complex with the rest of the ORC proteins. This is consistent with the observation in Chapter 3, that disruption of ORC stability also disrupts ORC subunit

phosphorylation. Prioritised phosphorylation of assembled ORC subunits could serve to enhance the stringency of re-licensing inhibition.

There is a rise in phosphorylation of a number of sites after S phase (biphasic substrates) involved in DNA replication related processes. These substrates, as discussed in Chapter 3, may be involved in the termination of replication and/or preparation of chromosomes for mitosis, post-S-phase. Following this, mitotic substrates are phosphorylated during G2/M. Of the mitotic substrates, those that are more sensitive to CDK activity are phosphorylated earlier during G2/M, suggesting that the orderly progression through mitosis is organised via the passage through sequential substrate specific CDK activity thresholds.

6.1 The function of CDK-mediated phosphorylation is unknown for many substrates

As discussed in the Introduction, CDK activity is important for the firing of origins upon S-phase initiation and the inhibition of origin licensing outside of G1. There is also a universal involvement of CDK in bipolar mitotic spindle assembly and attachment, chromosome condensation, phosphatase regulation, auto-regulatory CDK activation, as well as an array of other “typical” mitotic events (Introduction, section 1.2). Many CDK substrates defined here and by other large-scale approaches substantiate this picture (Holt et al., 2009). Numerous orthologs of *S. cerevisiae* Cdc28 substrates described in the literature are also novel *S. pombe* Cdc2 substrates. These include Rho-type GTPase activating proteins involved in polarised growth (Rga2, Rga3, Rga4, Rga7, Rga9), the Polo like kinase (Plo1), the DASH complex subunit Ask1, INCENP (Pic1), and the nucleoporin Nup40, amongst others (Enserink and Kolodner, 2010).

However, there are numerous CDK substrates, reported in Chapter 2, for which a CDK regulated function has not been reported. Some CDK substrates identified here may represent fission yeast specific substrates that have evolved to account for the idiosyncrasies of the life cycle, cell cycle and ecology of fission yeast. Some of these substrates have also been identified by phosphoproteomics as being phosphorylated downstream of CDK in *S. cerevisiae*, hinting at additional conserved regulatory functions for CDK, for which the molecular mechanism remains to be elucidated. For example, Msh6 and Dfp1 both show a biphasic behaviour in phosphorylation, peaking after S-phase, and are also both orthologs of Cdc28 substrates in budding yeast (Holt et al., 2009). Dfp1 activates the S-phase kinase Cdc7 (*S. pombe* Hsk1) (Labib, 2010) (i.e. Dfp1 is the ortholog *of S. cerevisiae* Dbf4) and in both organisms is phosphorylated by CDK in the disordered N-terminal region. The only detected CDK dependent site in *S. pombe* Dbf1 (T80), is immediately adjacent to two nuclear localisation sequences, hinting at a possible mode of action. The *S. cerevisiae* and *S. pombe* DNA mismatch repair protein, Msh6, is also phosphorylated in a predicted disordered N-terminal region

suggesting another conserved CDK regulated function in DNA metabolism. Fission yeast Rad4 (Dpb11 ortholog) is phosphorylated at mitosis whilst the BRCT domain-containing protein Mdb1 is a S-phase substrate pointing to additional instances of, as of yet, uninvestigated cross talk between the cell cycle machinery and the genome surveillance machinery.

Holt *e*t al. (2009) identified a number of protein involved in nuclear transport as Cdc28 substrates, two of which were also identified here as *S. pombe* CDK substrates (*S. pombe* Nup90 and Nup124) with multiple detected CDK dependent phosphorylation event that peaked in mitosis. Furthermore, phosphorylation events on *S. pombe* Nup40 and Nup146 are also dependent on CDK activity. This suggests a possible role for nucleopore regulation in the entry into, or execution of, mitosis. This is consistent with the observation that nucleopore function is required for timey mitotic entry in a dose dependent manner, as shown by the the fact that multiple *S. pombe* nucleoporin are haplo-insufficient for cell size (Jacqueline Hayles, personal communication). It has been proposed that CDK regulates the nuclear transport machinery at mitotic entry in human cell lines to accelerate nuclear import rates, and that Nup53 has also been defined as CDK substrates by *in vitro* covalent labelling of CDK substrates (Gavet and Pines, 2010a, Blethrow et al., 2008). The exact mechanism and importance of how the nuclear-cytoplasmic transport machinery changes during cell cycle transitions is not clear, but the fact that multiple nucloporins are CDK substrates suggests it merits further investigation.

CDK dependent phosphorylation of Rif1 was also identified in *S. pombe* and *S. cerevisiae*. Rif1 is involved in the regulation of telomere length, the selection and timing of origin firing and the repair of double strand breaks by non-homologous end joining. (Kumar and Cheok, 2014, Yamazaki et al., 2013). The interaction between the N-terminus of Rif1 and PP1 (Dis2) is important in antagonising DDK phosphorylation at late firing origins (Hiraga et al., 2014). *S pombe* Rif1-S50 phosphorylation peaks in mitosis, but how this phosphorylation relates to the known functions of Rif1 is unclear. S50 and a cluster of other proximate putative CDK sites are immediately adjacent to an NLS sequence and are situated between a S-I-L-K and R-V-X-F PP1 docking motif in the disordered Rif1 N-terminus, suggesting that CDK phosphorylation of Rif1 may release PP1 from Rif1 and/or regulate the

nuclear transport of Rif1 at the onset of mitosis. In addition to Rif1 there are a number of other telomere related proteins that are phosphorylated by CDK, including the telomere bouquet formation protein Bqt4 and telomere maintenance protein Ccq1.

The inositol polyphosphate phosphatase Inp53 is also a CDK substrate in both yeasts, whilst the *S. pombe* protein Lnp1 (the luna park ortholog, thought to play a role in tubular endoplasmic reticulum) and the inner nuclear membrane protein Lem2 are also CDK substrates during mitosis. The CDK regulation of transcription factors seems especially prevalent with multiple phosphorylation sites in Sep1, Atf1, and Adn2, as well as more than ten phosphorylation sites detected across other transcription factors including Cdc10 and Fhk2. There are also phosphorylation sites in two subunits of the INO80 chromatin remodeller complex, in a subunit of the HIRA complex (Hip1, involved in DNA synthesis independent histone deposition), and in a range of factors related to chromatin modification such as the Clr6 histone deacetylase associated PHD protein-1 Cph1, and Epe1 the putative H3-K9 demethylase.

The full list of CDK substrates defined here with the names of the human and *S. cerevisiae* orthologs and whether they were defined as having Cdc28-dependent phosphorylation in *S. cerevisiae* by Ubersax et al. (2003) and/or Holt et al. (2009) is listed in supplementary Sheet02.

6.2 What determines the differences in sensitivity to CDK between S-phase and mitotic substrates?

Multiple possible biochemical properties of CDK substrates could explain the differences in sensitivity to CDK activity, as discussed at length in Chapter 3 (section 3.5.1). The detailed characterisation of a handful of substrates will probably be required to elucidate the molecular principles that underpin these differences in behaviour. The observation that sites within the same protein behave similarly indicates that phosphorylation determinants are not all site autonomous. They could function at the level of the whole protein or simply at the level of a sub-protein domain. A simple test of this would be to investigate how the phosphorylation of a candidate S-phase substrate and a candidate mitotic substrate behave when the two are fused together in a single chimeric substrate. If either half of such a chimera were to be dominant over the other (i.e. the S-phase substrate causing premature mitotic substrate phosphorylated, or the mitotic substrate delaying the phosphorylation of the S-phase substrate moiety) it would suggest that protein wide determinants operate to specify the timing of a substrate. If this were the case, it would also provide a well-controlled system to properly integrate the nature of such determinants by screening for which fragments of the dominant moiety are required to alter the phosphorylation of the recessive moiety. Alternatively, if the S-phase and mitotic moiety of such a chimera were to behave independently it would indicate that the determinants for phosphorylation exist at the sub-protein domain level or lower, ruling out, for example, sub-cellular localisation as a determinant.

It is entirely plausible that numerous properties (e.g. docking motifs, domain structures, sub-cellular localisation) have differing significance in different substrates. Additionally, Cks1-dependent processive phosphorylation reactions could be necessary for the observation that sites in the same protein behave similarly. If a small number of sites in an S-phase substrate were more sensitive to CDK activity because of local sequence determinants, they could bind Cks1 once phosphorylated, positioning other less sensitive sites for enhanced phosphorylation by CDK *in cis*. Because Cks1 enables processive phosphorylation reactions there should be no detectable differences in the timing of phosphorylation of priming and

downstream sites. Such a scenario would allow a few sites to determine the phosphorylation of other CDK substrate sites, but would have to involve the appropriate spatial position of priming and downstream sites on the substrate. This could be simply tested by mutating CDK phosphorylated Threonines to Serines in candidate substrates, given that Cks1 can only bind phospho-threonine (Koivomagi et al., 2013).

The inter-dependencies of Orc1 and Orc2 phosphorylation illustrate that the determinants for specificity can operate at the level of an entire complex. There are other examples of multi-subunit complexes that include multiple CDK substrates that theoretically could behave similarly, such as the CPC. It is also worth considering whether larger sub-cellular structures, such as the nucleopore, the spindle, the SPB and the kinetochore, function as if they are one large substrate. For example, one or a small number of “adaptor” substrates could be required to determine the phosphorylation of other substrates at specific locations/structures within the cell. A systematic analysis of the inter-dependencies between different substrates would help elucidate any such substrate networks with “secondary substrates” organised around “primary substrates nodes” that would provide the defining biochemical determinants for phosphorylation timing. Although there is little data supporting such a model at present the hypothesis is attractive because of the large number of CDK substrates that exist within the cell.

6.3 Why are there multiple cyclins?

A significant body of work in other organisms has described the contribution of G1/S-phase cyclins as a combination of low activity Cyclin-CDK complexes with enhanced specificity towards certain substrates (see Introduction, section 1.4.1). The data presented in Chapter 4 suggest that *S. pombe* G1/S-phase cyclins form Cyclin-CDK complexes with lower activity than Cdc13-Cdc2, but do not provide any evidence of strong substrate specificity towards early substrates. To properly examine this CDK substrate phosphorylation should be quantified across a time course during the entry into and progression though a synchronised S-phase in the absence and presence of Cig1, Cig2 and Puc1. These and previous data are consistent with a model whereby S-phase is initiated by a larger number of less active Cyclin-CDK complexes that are, as such, better able to tune CDK activity around the lower threshold required for S-phase initiation, by resolving smaller differences in CDK activity. The low activity of G1/S-phase cyclins may also ensure that S-phase cyclins are never able to achieve sufficiently high CDK activity levels to initiate mitosis, helping to ensure the resolution of S-phase and mitosis, according to the quantitative model. Cdc13 is the only essential cyclin in fission yeast and there have been no successful attempts to re-engineer the expression or stability of other cyclins to complimenting *cdc13*Δ. If any of Cig1, Cig2 and Puc1 are indeed no different to Cdc13, other than being differentially expressed and producing lower CDK activity per Cyclin-CDK complex, they to should be able to drive the fission yeast cell cycle, under the appropriate expression conditions. Testing this will be important to further corroborating the quantitative model.

6.3.1.1 The evolution of cyclins and cell cycle complexity

Phylogenetic analysis of cell cycle-related cyclin orthologs across eukaryotes indicates that the cyclin complement, in fungi, contracted and re-expanded. *S. pombe* and *S. cerevisiae* contain only B-type cyclins whilst the last common ancestor to all modern day eukaryotes (LECA) almost certainly contained A, B & D-type cyclins, given that these cyclins are present from plants and algae through to animals (Cross et al., 2011). Fungi and animals are more closely related to each

other than they are to plants and form a monophyletic group: the opisthokonts. There are examples of basal fungi with both fungal cyclins and metazoan-related cyclins, whilst there are also basal fungi that have only a single fungal specific-type cyclin, and no other cell cycle related cyclins (Nicolas Buchler, personal communication). All metazoan-related cyclins were lost in the transition to Dikarya (which include the Ascomycota *S. pombe* and *S cerevisiae).* This could have occurred via the expansion of fungal cyclins, to compensate for loss of metazoan cyclins. Alternatively, it could have arisen via an evolutionary intermediary with only a single cyclin, from which the ancestor to modern day Dikarya expanded its cyclin repertoire by gene duplication and diversification. Why this shuffling, contraction and re-expansion of the cyclin complement has taken place is not clear. However, the flexibility within the fungal lineages to reshuffle their cyclin complement, through evolutionary time, could be for one of two reasons: selective advantages to adapting regulation i) upstream or ii) downstream of Cyclin-CDK complexes. Downstream changes could be brought about by the emergence of new cyclins to target a small handful of specific substrates (e.g. *S. cerevisiae* Clns). However, this would only involve the expansion, not the contraction or shuffling, of the cyclin compliment. For instance, the transition away from metazoan cyclins to fungal specific cyclins could have been driven by selective pressures to rework the pool of substrates involved in major cell cycle transitions. This seems unlikely given the conservation of CDK targets across species, and the presumed relative ease in evolving changes in a substrate as opposed to a cyclin (which has to account for many substrates simultaneously). Alternatively, new cyclins could have emerged to replace old ones to provide an novel regulatory setup. For example, the substrates of Cyclin-CDK could be unchanged, but an alerted profile or regulation of the CDK activity could offer a fitness advantage, perhaps via the ability to incorporate more or different environmental information into the decisions to undertake cell cycle transitions, consistent with fungal life as a free living, and sometimes single celled, organisms. This expansion of cyclins could have then provided a platform from which cyclin-substrate specificity may have arisen to refine and add robustness to the execution of cell cycle transitions.

It is worth re-iterating that CDK activity does not rise linearly during the cell cycle (Draetta and Beach, 1988, Labbe et al., 1988b, Moreno et al., 1989) and the acquisition of different cyclins, and their differential expression could have aided this. Although the non-linear dynamics of CDK activity accumulation are also due, to a large extent, to the autocatalytic switch like response in CDK activity at mitotic entry to activate CDK by turning off inhibitory T14 and Y15 phosphorylation (Novak and Tyson, 1993). In the minimal CDK network where endogenous cyclins are deleted, cells can tolerate the phospho-mutation of T14 and Y15 (Coudreuse and Nurse, 2010). This could suggest that either, cells can appropriately temporally resolve all necessary thresholds when CDK activity accumulates linearly, or that additional mechanism are operating to uncouple cyclin accumulation from CDK activity. The synthetic lethality of this background with the deletion of the stoichiometric inhibitor of CDK, Rum1, or the major catalytic subunit of the PP2A phosphatase (Pp2a) is consistent with the latter (Navarro and Nurse, 2012, Coudreuse and Nurse, 2010).

It has been proposed that quantitative model represented an ancestral eukaryotic state with a single Cyclin B-CDK-APC/C oscillator organising the cell cycle (Nasmyth, 1995). However the fact that the last common ancestor to all modern day eukaryotes (LECA) contained A, B & D-type cyclins (Cross et al., 2011) suggests that the LECA had acquired a cell cycle network as complex as modern day metazoa using multiple Cyclin-CDK complexes. This is also true of other eukaryotic attributes, such as the presence of mitochondrial genes, suggesting that the inferred properties of LECA may not represent that of a truly primitive or nascent eukaryote (Embley and Martin, 2006). Given that the cell cycle machinery present in LECA may not necessarily reflect that of the earliest eukaryotes it is an open question as to what regulatory principles underpinned the initial evolution of the Cyclin-CDK regulation of the cell cycle. A reasonable speculation would be that, in response to a selective advantage for the separation of DNA replication and chromosome segregation, the rising activity of a single kinase could have organised cell cycle events via differential substrate phosphorylation. This selective pressure would have presumably been related to an increase in genome size and complexity. The subsequent complexity that evolved in LECA could still have been underpinned by a simple CDK activity threshold model, as supported by the ability

of modern day eukaryotes (i.e. fission yeast) to drive the cell cycle with a single Cyclin-CDK. The inability to similarly strip down the cell cycle machinery of numerous other eukaryotes could be rationalised by two extreme views. Either they no longer operate on a predominantly quantitative model and are dependent on qualitative differences in cyclin specificity to execute the orderly progression through the cell cycle. Alternatively, no attempts to run a cell cycle from a single Cyclin-CDK complex has managed to recapitulate the appropriate activity profile, normally the product of multiple Cyclin-CDK complexes, with a single Cyclin-CDK complex.

6.4 Testing the universality of the quantitative model

In higher eukaryotes different cyclins have the ability to complement each other and re-engineered Cyclin B1 constructs can be used to initiate DNA replication, as well as mitotic entry (see Introduction, section 1.4.3) (Moore et al., 2003). Furthermore only a single CDK; Cdk1, is required for cell division (Santamaria et al., 2007). These observations are consistent with the conservation of the quantitative model. However, directly testing the quantitative model in higher eukaryotes, where other regulatory layers confound the core organising mechanism of cell cycle progression, could prove experimentally complex. A simple way to address this would be to ask two questions about vertebrate proteins ectopically expressed in *S. pombe*. Firstly, can any vertebrate Cyclin-CDK, like Cdc13-Cdc2, preferentially phosphorylate *S. pombe* S-phase substrates over mitotic substrates? Secondly, are vertebrate S-phase substrates more sensitive to CDK activity than mitotic substrates in *S. pombe*? If the answer to both of these questions were yes, then it would support the notion that higher eukaryotic Cyclin-CDK activity orders the cell cycle as per the quantitative model. If a vertebrate Cyclin-CDK was able to differentially phosphorylate S-phase and mitotic substrates, and was able to acquire the appropriate profile of CDK accumulation, then such a vertebrate Cyclin-CDK should be able to drive the fission yeast cell cycle in the absence of endogenous *S. pombe* CDK or cyclins. The complementation of *S. pombe cdc2* mutant alleles with human Cdk1 was first reported in 1987 (Lee and Nurse, 1987), but there has been no reported attempt to complement fission yeast *cdc13* with any other cyclins. One possible explanation for this could be the dynamic spatial regulation that Cyclin B1 is subjected to in higher eukaryotes, meaning it never even reaches the nuclear compartment in fission yeast. A possible way forward would be to re-engineer aspects of Cyclin B regulation or to undertake a saturating mutagenic screen for Cyclin B mutants that complement *cdc13*.

6.5 Concluding remarks

The cell cycle network controls some of the most critical decision making process in cell biology. The molecular composition of the cell cycle control network is becoming increasingly well described but some of the major outstanding issues in the field seem to relate to the system properties and quantitative behaviour of these networks. That is to say, a reasonable amount is known about “who” makes the some of these decisions, but rather less about “how” and “why”.

Kinases, and thus phosphorylation, sit at the heart of cell cycle control. As such, understanding the relationship between the major regulatory cell cycle kinases, such as CDK, and the dynamics of their substrate phosphorylation is a critical aspect of understanding the system and quantitative properties of cell cycle control. The work presented in this thesis supports the view that the directionality and order in the cell cycle is achieved predominantly by properties of CDK substrates that impart differential sensitivity to a rising profile of CDK activity.

The major outstanding questions raised by this thesis are what substrate properties determine CDK substrate phosphorylation timing and what are the critical system properties (i.e. CDK activity dynamics and substrate sensitivity) that are conserved across eukaryotes that are central to ensuring proper cell cycle progression.

Chapter 7. Materials and methods

7.1 Cell culture and *S. pombe* genetics

S. pombe media and methods are as previously described (Moreno et al., 1991). Strains are listed in Sheet01 (sheet01_experiment and strain list) and Table 7-1. The following constructions are previously reported: ∆*CCP* (Martin-Castellanos et al., 2000)*, cdc13(as)-L-cdc2 ∆2 ∆13 ∆CCP, cdc13-L-cdc2AF(as) ∆2 ∆13 ∆CCP* (Coudreuse and Nurse, 2010)*, lys3-37, arg1-230 car2∆* (Bicho et al., 2010), *sld3-5flag* (Fukuura et al., 2011), *nsk1-GFP* (Chen et al., 2011), *orc1-HA* (Grallert and Nurse, 1996)*, rad3∆* (Kim et al., 2010)*, cdc2(as)M17* (Aoi et al., 2014), *orc2-2* (Kiely et al., 2000), *orc1-4* (Grallert and Nurse, 1996). C-terminal tagging (*orc2-3pk*) and gene deletion cassette switching was performed as previously reported (Bahler et al., 1998b). For ectopic expression of C-terminally 5pk tagged *drc1*, *sld3* and *orc2*: synthetic CDS (Integrated DNA technologies (gBlocks) and Life Technologies (GeneArt Strings)) were subcloned into RIP3X with an attenuated (nmt41) promoter (Basi et al., 1993, Maundrell, 1993). PacI linearised plasmids were transformed integrated at the nmt1 locus (ScLeu2 selection). Derivatives of the above were constructed by crosses and confirmed by marker selection and/or colony PCR.

All experiments are performed in exponential growth. All experiment for proteomics were performed in SILAC media (EMM (6 nM ammonium chloride) + 0.25 mg/ml leucine, 0.15 mg/ml uridine, 0.04 mg/ml arginine and 0.03 mg/ml lysine) (Bicho et al., 2010)**.** For all heavy labelled samples (H) cells were grown in exponential growth for >8 generations in SILAC media supplemented with heavy arginine (L-arginine:HCL (U13C6, 99%)) and heavy lysine (L-lysine:2HCL (U13C6, 99%)) isotopes (Cambridge Isotope Laboratories Inc.). All other experiments are performed in EMM4S except where the use of YE4S media is specified (Moreno et al., 1991). G2 cell cycle arrests were imposed during exponential growth using 1 µM 1-NmPP1 treatment for one generation. Times (min) after wash and release are with respect to the initial re-suspension of cells during the first of three washes.

DNA content was determined by flow cytometry (FACS). Ethanol (70%, v/v) fixed cells were washed and resuspended in 50 mM sodium citrate before incubation with RNase A (0.1 mg/ml) (SIGMA) (>12 h, 37 °C). DNA was stained with propidium iodide (2 µg/ml) (SIGMA) before sample sonication. DNA content per cell was acquired for 10,000 events on a BD LSR-Fortessa. DNA content is displayed on a log scale after gating for single, whole cells in FlowJo X (Treestar Inc).

Nuclear division and cell division was scored in heat fixed samples by monitoring DNA (DAPI, SIGMA) and septum formation (calcofluor, SIGMA), respectively (Zeiss Axioskop microscope, 63×/1.4 NA objective) (cell photos taken with a QICAM Fast Digital Camera).

7.2 Protein extractions and Western blotting

Cell cultures were quenched by adding 100% (w/v) ice cold trichloroacetic acid to a final concentration of 10%. Cells were kept on ice for >20 min, pelleted and washed in acetone. Cell pellets were then washed and resuspended (100µl) in Lysis buffer (8M urea & 50mM ammonium bicarbonate + cOmplete Mini EDTA-free protease inhibitor cocktail (Roche) + PhosSTOP phosphatase inhibitor cocktail (Roche)). 1.2 ml acid wash glass beads (0.4mM, SIGMA) were added to cell suspensions, which were then beaten (FastPrep120) to beak cells. Cell debris was pelleted and supernatant was recovered as a protein extract (stored at -80 °C). Samples used for Lambda phosphatase assay (NEB) were extracted as above with the omission of PhosSTOP)

Phosphorylation dependent band shifts in Orc1, Orc2, Sld3 and Bir1 were resolved using Phos-tag (Alpha Laboratories) supplemented SDS-PAGE under neutral pH conditions (Kinoshita and Kinoshita-Kikuta, 2011). Protein detection by Western blotting was performed using primary antibodies as follows. Cdc13-L-Cdc2: 1:6,000 SP4 antibody (rabbit polyclonal) (Moreno et al., 1989). Cdc2-Y15P: 1:500 phospho-Cdc2 (Tyr15) antibody (rabbit polyclonal, #9111 Cell Signalling Technology). Alpha tubulin: 1:10,000 TAT1 antibody (mouse monoclonal) (Woods et al., 1989). Bir1: 1:1,000 anti-Bir1 antibody (rabbit polyclonal) (Tsukahara et al., 2010). Dis2: 1:1,000 anti-Dis2 antibody (rabbit polyclonal) (63-119, Bioacedemia). Dis2-T316P: 1:1,000 anti-Dis2-T316P (rabbit polyclonal) (63-121 Bioacedemia). Pk epitope tag: SV5-PK1 (mouse monoclonal, 1:1,000) (MCA1360, AbD seroTEC). HA epitope tag: 1:1,000 anti-HA-Tag C29F4 antibody (rabbit polyclonal) (#3724, Cell Signaling Technology). Flag epitope tag: 1:1,000 ANTI-FLAG M2 antibody (mouse monoclonal) (F3165, SIGMA). Secondary antibodies: horse radish peroxidase-conjugated donkey anti-rabbit (NA934V, GE healthcare) or goat anti-mouse (STAR120P, abD SeroTEC) (1:25,000). Signal was detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) or SuperSignal West Femto Maximum Sensitivity Substrate (34095, Life Technologies) and imaged using ImageQuant LAS 4000. Quantification of Western blots was performed using ImageQuant TL software (GE Healthcare).

7.3 Sample preparation for mass spectrometry

All heavy or light samples were mixed with a light or heavy reference samples respectively. Samples were mixed in 1:1 protein amount ratios (protein concentration determined by Bradford assay). All SILAC experiments and sample names are listed in Sheet01 (sheet01_experiment and strain list). Members of The Francis Crick Institute Protein Analysis and Proteomics facility carried out sample preparation and data acquisition as follows. Samples were reduced with 1 M dithiothreitol (DTT) (25 min, 56 °C), alkylated with 500 mM iodoacetamide (30 min, rt, dark) and quenched with 1 M dithiothreitol. Samples were then diluted with 50 mM ammonium bicarbonate to reduce the urea concentration to <2 M, prior to trypsin digestion (37 °C, overnight). Peptides were acidified to 0.4% with trifluoroacetic acid (TFA) and centrifuged (14krpm at 4 °C for 30 min). Peptides were then desalted using a C_{18} SepPak Lite, 130 mg bed volume, under vacuum and dried. Peptides were then digested using Lys-C in 10% acetonitrile, 50 mM ammonium bicarbonate (37 °C, 2 h), followed by trypsin digestion (37 °C, overnight). Digested peptides were then again desalted and dried.

Titanium dioxide enrichment was then carried out as follows. Dried fractions were re-suspended in 80% acetonitrile, 5% trifluoroacetic acid, 1 M glycolic acid, sonicated (10 min) and added to 5 mg of titanium dioxide beads. The beads were washed using 80% acetonitrile and 1% trifluoroacetic acid followed by 10% acetonitrile and 0.2% trifluoroacetic acid, and dried under vacuum centrifugation. For analysis of non-phosphorylated peptides, flowthroughs were retained. Phosphopeptides were then eluted from the beads by adding 1% ammonium hydroxide followed by 5% ammonium hydroxide, and dried by vacuum centrifugation. Dried phosphopeptides were then re-suspended in 100 µl of 1% trifluoroacetic acid and sonicated (15 min). A C_{18} membrane was packed into a 200µl pipette tip and washed using methanol and equilibrated with 1% trifluoroacetic acid. The peptides were then loaded onto the Stage Tip and washed with 1% trifluoroacetic acid followed by elution with 80% acetonitrile, 5% trifluoroacetic acid. The eluted peptides were again dried under vacuum centrifugation. For analysis of non-phosphorylated peptides, the stored flowthroughs were dried, desalted using a C_{18} SepPak Lite, 130 mg bed volume, under vacuum and dried. Peptides were then separated into 12 fractions using strong cation exchange (SCX) liquid chromatography, and dried, if necessary, to 250 µl.

For LC-MS/MS data acquisition analysis, the following was performed. Phosphopeptide mixtures were re-suspended in 35 µl 0.1% trifluoroacetic acid, and injected three times (10 µl per injection). Non-phosphopeptide mixtures were diluted 1:10 (v/v) in 0.1%

trifluoroacetic acid, and injected three times (10 µl per injection). Each run consisted of a 3 h gradient elution with one activation method per run: CID, MSA and HCD. An LTQ-Orbitrap Velos was used for data acquisition of phosphopeptides, and an LTQ-Orbitrap Velos Pro was used for data acquisition of non-phosphorylated peptides.

Data processing was performed using the MaxQuant bioinformatics suite as adopted by the Crick Protein Analysis and Proteomics facility, and protein database searching was performed by the Andromeda search engine using a UniProt database of *S. pombe* proteins amended with common contaminants. For all analyses (besides CCC6773) the UniProt database of *S. pombe* proteins was modified to account for the altered genetic background of the reference sample (P0000 = Cdc13-L-Cdc2). Default MaxQuant parameters were used with the following adjustments: Phospho (STY) was added as a variable modification (for the phospho-samples only), Lys6 and Arg6 were the heavy labels, 'Filter labelled amino acids' was deselected, minimum ratio count was set as two, re-quantify was selected with the instruction to keep low-scoring versions of identified peptides within parameter groups, and match between runs was selected. Experiments groups in which raw data was search are listed in Sheet01 (sheet01_experiment and strain list).

7.4 Analysis of mass spectrometry data

Members of the Francis Crick Institute Protein Analysis and Proteomics facility assisted with data analysis only where stated.

Phospho STY.txt (phosphopeptides) and ProteinGroup.txt (peptides) MaxQuant outputs were uploaded into and analysed in Pereus1.4.0.2. Normalised H/L ratios were used for relative site phosphorylation and protein levels respectively. Where only singly phosphorylated peptides were analysed: PhosphoSTY_1 output were imported into Pereus1.4.0.2 instead. All linear regressions, non-linear curve fitting (sigmoidal function and exponential decay), AUC (Area Under Curve) calculations, student t-tests and binomial test were performed in Prism 6 on normalised H/L ratio exported from Pereus1.4.0.2.

7.4.1 Defining CDK substrates and calculating one phase decay parameters

CCC5977 & CCC5978 was filtered for phosphorylation events at S/T-P (minimal CDK consensus site) with a localisation probability (loc. prob.) > 0.9. Then the following 4 criteria were used to define CDK substrates in mitosis (CCC5978) or S-phase (CCC5977).

i) M00 is a null distribution (i.e. heavy and light samples are equivalent), so all H:L values should theoretically be 1. As such the dataset was filtered for phosphorylation sites for which M00 deviated less than twofold from H:L=1. S00 was filtered for sites identified in S-phase with a H:L ratio greater than 0.25. (i.e. peptides for which a ratio was quantified but the peptide(s) was only detected in the light reference (ISO-MATCH) were excluded). *ii)* Phosphorylation events with less than 4 valid ratios were excluded. *iii)* Sites that had at least one ratio less than half the value of the initial time point ratio were kept. *iv)* A non-linear one-phase decay was fitted to the data using the least squares (ordinary) fitting method (Prism 6). The curve was constrained such that the K > 0 and Plateau > 0. CDK substrates were defined as sites that fitted an exponential decay with R squared > 0.9 and a plateau < 0.5. *v)* Sites that did not pass the above criteria were screened for anomalous ratios as follows. The dataset was duplicated six times. Within each duplicate, one time point (except for 0 min from CDK inactivation (M00min & S00min) was removed. Each modified dataset was re-analysed by the four above criteria (*i-iv*). If a phosphorylation site in one of the six modified datasets was now defined as a substrate it was included as a substrate with the respective modified dataset values used to calculate all subsequent parameters (e.g. half life). Where multiple deletions independently permitted a site to pass the four above criteria, the one that gave the highest R squared value was used. Anomalous points are likely due to technical considerations such as the use of different species to quantify site phosphorylation at different time points (e.g. 31.1% of sites with an anomalous point(s) in the profile of dephosphorylation in mitosis satisfy the above criteria when only singly phosphorylated peptides were analysed before anomaly screening).

7.4.2 Consensus sequences and annotation enrichments

Consensus sequence surrounding phosphorylation events were generated in Perseus 1.4.0.2. Annotation (including gene ontologies) enrichment was performed in Perseus 1.4.0.2. 2D annotation enrichment (entire phosphoproteome) was performed for M12min and M24min in CCC5978 (loc. prob. > 0.9). Enrichment of CDK substrates was performed against all sites in CCC5977&CCC5978 (loc. prob. > 0.9) by Fisher exact test (B-H FDR < 0.02). Enrichment of motifs and annotation (including gene ontologies) categories in Clusters A-H (Figure 5.02) was performed in by Fisher exact test (B-H FDR < 0.02) against non S/T-P sites in CCC6254 after missing values were replaced by imputation (loc. prob. > 0.9).

7.4.3 Imputation and smoothing

Imputation was applied to replace missing values. Only phosphorylation sites or protein groups with at least 50% valid values quantified, within the respective dataset, where analysed. The number of sites/proteins to which imputation was applied and the number of values imputed in the respective datasets are listed in Sheet01. Less than 15% of any dataset after imputation, constituted imputed values. Missing values were imputed using the R package DMwR (Torgo, 2010). Default parameters of knnImputation function were used (The Francis Crick Institute Protein Analysis and Proteomics facility). Imputed data is only used where stated. Smoothing was applied to imputed data as listed in Sheet01. An R script was applied in which five nearest neighbours for each site were identified by Euclidian distance. The mean value of these five nearest neighbours ratios was then substituted for each original ratio (The Francis Crick Institute Protein Analysis and Proteomics facility). Smoothed data is only used when stated.

7.4.4 Principle component analysis (PCA) and hierarchical clustering analysis

PCA and hierarchical clustering was performed on cell cycle dependent phosphorylation or protein levels, with missing values imputed (as above), in Perseus 1.4.0.2 (PCA settings: default. Clustering settings: cluster rows, euclidian distance, do not presuppose K-means).

7.4.5 Secondary structure analysis

DisEMBL algorithm was applied to categorise all potential Serine, Threonine and Tyrosine site in the *S. pombe* proteome into disordered or not disordered (The Francis Crick Institute Computational biology facility). One tailed binomial test was used to test the significance of difference between datasets.

7.4.6 Calculation of substrate sensitivity to CDK activity

Non-linear sigmoidal curve (four parameter logistic function) was fitted to the data (relative phosphorylation (L:H) across a range of 1-NmPP1 concentrations) using the least squares (ordinary) fitting method (Prism 6). The curve was constrained such that the Hill slope < 0 and Bottom > 0. Outlier detection was used (Q = 10%). Sites with fitted curves (R square > 0.9, Bottom < 0.5 and Top/Bottom > 2) were used to derive IC50 and Hill slope values.

7.5 Supplementary electronic files

An electronic copy of this thesis along with three supplementary excel spreadsheets (Sheet01-03) are stored on a CD-ROM attached to the inside cover of this thesis.

Strain ID	mating type	Genotype
MS86	h (+?)	car2Δ::hphMX6_arg1-230_lys3-37_leu1+_leu1Δ::Pcdc13::cdc13-L-cdc2AF (as)::cdc13 3'UTR::ura4+_cdc2Δ::KanMX6 _cdc13Δ::natMX6_cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS87	h (+?)	rad3Δ::bsdMX6_car2Δ::HphMX6_arg1-230_lys3-37_leu1+_leu1Δ::Pcdc13::cdc13-L-cdc2AF (as)::cdc13 3'UTR::ura4+ _cdc2Δ::KanMX6_cdc13Δ::natMX6_cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS108	h +	leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::kanMX6_cdc13Δ::natMX6_cig1Δ::ura4+_cig2Δ::ura4+ _puc1Δ::ura4+_ura4-D18
MS122	h +	car2Δ::hphMX6_arg1-230_lys3-37_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::KanMX6 _cdc13Δ::natMX6_ura4-D18
MS131	h +	cdc2-asM17::bsdMX6_car2Δ::hphMX6_arg1-230_lys3-37
MS132	h +	Sld3-5flag::ura4+_orc2-3pk::hphMX6_nsk1-GFP::kanMX6_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+ _cdc2Δ::scLeu2_cdc13Δ::natMX6_(ura4-D18?)
MS200	h +	cdc2-asM17::bsdMX6_car2Δ::hphMX6_arg1-230_lys3-37_cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS212	h (+?)	sld3-5flag::ura4+_orc2-3pk::hphMX6_Nsk1-GFP::KanMX6_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+ _cdc2Δ::scLeu2_cdc13Δ::natMX6_cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_(ura4-D18?)
MS213	h (+?)	car2Δ::hphMX6_arg1-230_lys3-37_leu1+_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::KanMX6 _cdc13Δ::natMX6_cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS230	h +	car2Δ::hphMX6_arg1-230_lys3-37_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::KanMX6 _cdc13Δ::natMX6_cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS283	h (+?)	orc2-2(ts)_orc1-HA leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::ScLEU2_cdc13Δ::natMX6 _(ura4-D18?)
MS284	h (-?)	orc2-3pk::hphMX6_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::kanMX6_cdc13Δ::natMX6 _(ura4-D18?)
MS285	h (+?)	orp1-4(ts)_sld3-5flag::ura4+_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::scLeu2_cdc13Δ::natMX6 _(ura4-D18?)
MS286	h (+?)	orc2-2(ts)_sld3-5flag::ura4+_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::scLeu2_cdc13Δ::natMX6 _(ura4-D18?)
MS395	h +	nmt1::nmt41-drc1wt-5pk_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::kanMX6_cdc13Δ::natMX6 _cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS396	h +	nmt1::nmt41-drc1AGGA-5pk_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::kanMX6_cdc13Δ::natMX6 _cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS397	h +	nmt1::nmt41-orc2wt-5pk_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::kanMX6_cdc13Δ::natMX6 _cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS398	h +	nmt1::nmt41-orc2AGGA-5pk_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::kanMX6_cdc13Δ::natMX6 _cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS399	h +	nmt1::nmt41-sld3wt-5pk_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::kanMX6_cdc13Δ::natMX6 _cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18
MS400	h +	nmt1::nmt41-sld3AGGA-5pk_leu1Δ::Pcdc13::cdc13-L-cdc2(as)::cdc13 3'UTR::ura4+_cdc2Δ::kanMX6_cdc13Δ::natMX6 _cig1Δ::ura4+_cig2Δ::ura4+_puc1Δ::ura4+_ura4-D18

Table 7-1 | *S. pombe* strain list

Reference List

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